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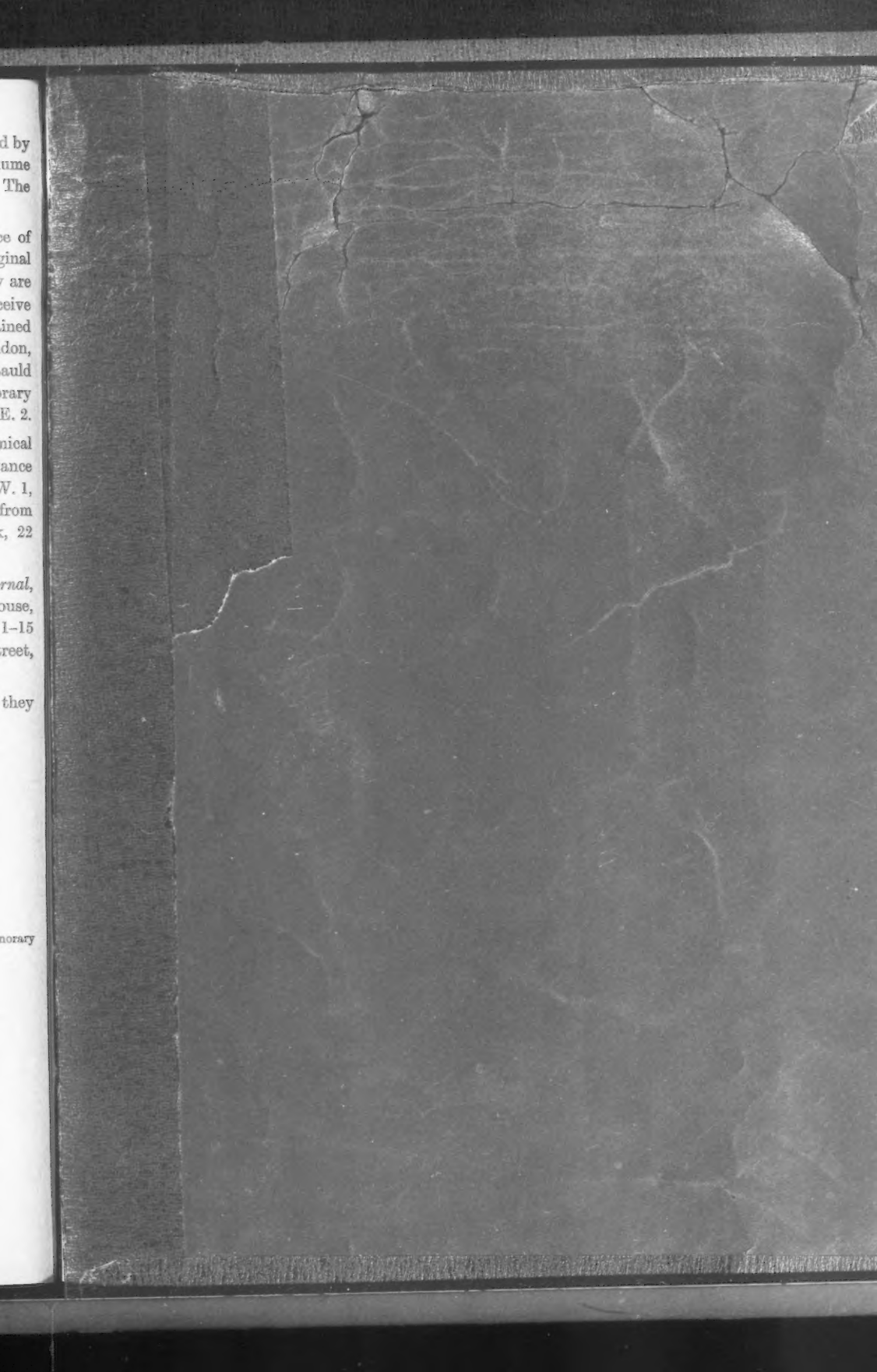

The Fatty Acid Composition and Glyceride Structure of Indian Wild-boar Fat

By S. P. PATHAK, S. K. ROY and B. N. TRIVEDI
Department of Chemical Engineering and Chemical Technology, Banaras Hindu University, Varanasi, India

(*Received* 12 *May* 1958)

Only a few land-animal fats have been studied in detail so far with respect to their fatty acid composition and the pattern of glyceride molecules. Among those whose fatty acid compositions are available are lion and cat (Hilditch, Sime & Maddison, 1942; Gunstone & Russell, 1954), puma (Giral, 1945), tiger (Pathak & Agarwal, 1952; Gunstone, 1955), sacred baboon, Ceylon sloth bear (Hilditch *et al.* 1942), badger (Gupta, Hilditch & Meara, 1950), man (Cramer & Brown, 1951; James & Wheatley, 1956), horse, camel, deer, ox, sheep and pig (Hilditch, 1956), representing carnivorous, omnivorous and herbivorous types of animals. The present investigation of the component acids and glyceride structure of Indian wild-boar depot fat was undertaken to add more data to the scanty literature on herbivorous-animal fats.

EXPERIMENTAL

A colourless fat was obtained from the upper left thigh region of the wild boar (male) of the forest of Hamirpur (Northern India). The saponification equivalent (S.E.) of the purified fat was 282·6 and iodine value (I.V.) was 50·8 with 2·7% of free fatty acid (as oleic).

Component acids. A portion (92 g.) of the fat was saponified and the mixed fatty acids (87 g.; I.V. 52·4) were recovered; the mixed acids so obtained were resolved by the lead salt–ethanol method (Hilditch, 1956) into two groups of simpler mixtures of acids differing in unsaturation, whereupon each group of the acid was esterified with methanol, with the precautions suggested by Bjarnason & Meara (1944), and fractionated through the electrically heated and packed (E.H.P.) column of Longenecker (1937) as usual. The composition of the mixed acid was calculated from the S.E. and I.V. values of the ester subfractions by the method described by Hilditch (1956). The mean equivalent of each homologous ester group was found by interpolation and extrapolation of the degree of unsaturation of each fraction.

Component glyceride. A sample (221 g.) of the neutral fat (I.V. 48·9) was dissolved in acetone (2·2 l.) and kept at 10°, when 41·8 g. (I.V. 28·8) of the glyceride crystallized out. The soluble portion, 179·2 g. (I.V. 54·8), was again kept in acetone (1·8 l.) at 0°, which deposited 33·5 g. (I.V. 35·8) of glyceride. The soluble portion of glyceride in acetone at 0°, 145·7 g. (I.V. 59·1), was further crystallized in acetone (1·5 l.) at −10°, and 27·3 g. (I.V. 43·2) of glyceride separated out and 118·4 g. (I.V. 62·8) of acetone-soluble glyceride at −10° was recovered separately. The glycerides precipitated in acetone at 0° were dissolved in ether (0·2 l.) and cooled to −5°, when 6·2 g. (I.V. 31·4) of ether-insoluble glyceride was obtained. The ether-soluble glyceride, 27·3 g. (I.V. 36·8) at −5°, was recovered separately.

The fractions thus obtained were combined with respect to the nearly similar iodine values. Thereby three fractions, 48 g. (I.V. 29·1), 54·6 g. (I.V. 40·0) and 118·4 g. (I.V. 62·8), were formed and named A, B and C respectively. The mixed acids recovered from each glyceride fraction were resolved into solid (A) and liquid (B) acid groups by the lead salt–ethanol method. These resolved fractions were called AA, AB, BA, BB, CA and CB respectively. Each fraction was converted into methyl ester and then fractionated through the E.H.P. column as usual.

The possible component glycerides were calculated from the composition of the individual group on the theory of computation given by Hilditch (1956).

RESULTS

The fractional crystallization data of the mixed fatty acids of wild boar by the lead salt–ethanol method are given in Table 1.

Table 2 records the component acids of wild-boar fat.

The results obtained by crystallization of neutral wild-boar fat from acetone and ether at various temperatures are recorded in Table 3.

Table 4 presents the separation of the mixed acids of glyceride fractions A, B and C by the lead salt–ethanol method.

The component acids of the glyceride fractions A, B and C are given in Table 5.

The possible component glycerides of wild-boar fat are given in Table 6.

DISCUSSION

Component acids. The wild-boar fat contains 47·9% by weight of the saturated acids, which includes 29·5% of palmitic and 16·8% of stearic acid as major components and 1·6% of myristic acid as a minor part. The unsaturated acids, which form 52·1% of the total acids, contain 13·2% of hexadecenoic and 36·3% of oleic acid together with 0·7% of tetradecenoic and 1·9% of linoleic acid. This is, in general, similar to the pattern in other fats of other herbivorous land animals.

Table 1. *Separation of the acids of wild-boar fat by the lead salt–ethanol method*

Group	Solubility of lead salt in ethanol at 15°	Weight (g.)	Weight (%)	Iodine value
A	Insoluble	34·2	40·2	7·5
B	Soluble	50·8	59·8	82·6

Table 2. *Component acids in groups A and B from the whole fat of the wild boar*

Figures in parentheses indicate unsaturation, e.g. −2·0 H means monoethenoid.

Acids	Component acids* of groups (%) A	Component acids* of groups (%) B	Total (%)	Fatty acids in the whole fat excluding non-saponifiable fraction % (w/w)	Fatty acids in the whole fat excluding non-saponifiable fraction % (mol.)
Saturated					
Myristic	0·5	1·1	1·6	1·6	1·9
Palmitic	25·8	3·6	29·4	29·5	31·0
Stearic	10·7	6·1	16·8	16·8	15·9
Unsaturated					
C_{14}	—	0·8 (−2·0 H)	0·8 (−2·0 H)	0·8	0·9
C_{16}	1·4 (−2·0 H)	11·8 (−2·0 H)	13·2 (−2·0 H)	13·2	14·0
C_{18}	1·7 (−2·0 H)	36·3 (−2·2 H)	38·0 (−2·2 H)	38·1	36·3
Unsaponifiable	0·1	0·1	0·2	—	—

* Figures are derived from the quantities of fractions actually obtained on fractionation; loss was little and was ignored. Hence the figures total 100.

Table 3. *Crystallization of neutral wild-boar fat from acetone and ether*

Fraction	Description*	Weight (g.)	Weight (%)	Iodine value
A	Glycerides insoluble in acetone (10) at 10° and 0° and insoluble in ether (5) at −5°	48·0	21·7	29·1
B	Glycerides soluble in acetone (10) at 10°, insoluble at 0° and soluble in ether (5) at −5° but insoluble in acetone (10) at −10°	54·6	24·7	40·0
C	Glycerides soluble in acetone (10) at 10°, 0° and −10°	118·4	53·6	62·8

* Figures in parentheses represent ml. of acetone and ether/g. of fat.

Table 4. *Separation of the mixed acids of glyceride fractions A, B and C*

Fraction A	Solubility of lead salt in ethanol at 15°	Weight (g.)	Weight (%)	Iodine value
AA	Insoluble	26·8	63·8	1·2
AB	Soluble	15·2	36·2	79·9
Fraction B				
BA	Insoluble	25·5	53·1	4·9
BB	Soluble	22·5	46·9	82·6
Fraction C				
CA	Insoluble	26·5	25·0	2·7
CB	Soluble	79·5	75·0	83·8

Table 5. *Component acids of glyceride fractions A, B and C (% mol.)*

Component acids	A	B	C	Total
Myristic	0·2	0·6	1·6	2·4
Palmitic	8·6	9·7	13·9	32·2
Stearic	6·1	3·3	3·6	13·0
Tetradecenoic	0·1	0·4	0·8	1·3
Hexadecenoic	1·7	1·9	8·7	12·3
Oleic	5·0	8·4	22·5	35·9
Linoleic	—	0·4	2·5	2·9

Table 7 shows that the wild-boar fat and pig fat are extremely similar in their composition. The same saturated and unsaturated acids, except C_{20}–C_{22}, are present in each case. The characteristic difference between wild-boar fat and pig fat is that the wild-boar fat has a larger amount of hexadecenoic acid, formed mostly at the expense of oleic and octadecadienoic acid, whereas pig fat has a lower percentage of hexadecenoic acid but the

Table 6. *Component glycerides of wild-boar fat*

	A	B	C	Whole fat
Component acid groups (% mol.)				
Myristic and palmitic	8·8	10·3	15·4	34·5
Stearic	6·1	3·3	3·6	13·0
Tetradecenoic and hexadecenoic	1·7	2·4	9·5	13·6
Oleic and linoleic	5·0	8·8	25·1	38·9
Component glyceride groups (% mol.)				
(*a*) Fully saturated group	1·5	—	—	1·5
Disaturated–mono-unsaturated	20·1	16·0	3·5	39·6
Monosaturated–diunsaturated	—	8·8	50·1	58·9
(*b*) Dipalmito-mono-others	4·8	6·1	—	10·9
Monopalmito-di-others	16·8	18·7	46·3	81·8
Tri-others	—	—	7·3	7·3
(*c*) Dioleo-mono-others	—	1·6	21·6	23·2
Mono-oleo-di-others	18·2	23·1	32·0	73·3
Tri-others	3·5	—	—	3·5
Possible component glycerides (% mol.)				
Fully saturated (1·5%)				
Myristodipalmitin	0·6	—	—	0·6
Dipalmitostearin	1·0	—	—	1·0
Disaturated–mono-unsaturated (39·6%)				
Dipalmitohexadecenin	3·2	—	—	3·2
Dipalmito-olein	—	6·1	—	6·1
Palmitostearohexadecenin	2·0	—	—	2·0
Palmitostearo-olein	14·9	9·9	3·5	28·3
Monosaturated–diunsaturated (58·9%)				
Hexadeceno-oleopalmitin	—	7·1	17·8	24·9
Hexadeceno-oleostearin	—	—	10·7	10·7
Palmitodiolein	—	1·6	21·6	23·2

Table 7. *Component acids (% by wt.) of pig and wild-boar fats*

Acids	Wild boar (present work)	Pig (back, outer) (Hilditch, Lea & Pedelty, 1939)	Pig (back, inner) (Hilditch *et al.* 1939)
Myristic	1·6	1·3	1·0
Palmitic	29·5	28·3	30·1
Stearic	16·8	11·9	16·2
Tetradecenoic	0·7	—	—
Hexadecenoic	13·2	2·7	2·7
Oleic	36·3	47·5	40·9
Octadecadienoic	1·9	6·0	7·1
C_{20}–C_{22}	—	2·1	1·7

Table 8. *Distribution of acyl groups in the glyceride of wild-boar fat*

		Computed	
	Actual	Even (Hilditch, 1956)	Random (Kartha, 1949, 1953)
Fully saturated glyceride	1·5	0	10·7
Disaturated–mono-unsaturated	39·6	42·6	35·6
Monosaturated–diunsaturated	58·9	57·4	39·3
Triunsaturated	0	0	14·4

unsaturated acids octadecadienoic and C_{20}–C_{22} acids are in higher proportion in pig fat, showing more unsaturation than the wild-boar fat. There is thus a generic similarity between domestic and wild varieties of the animal, which is a usual feature in both animal and vegetable fats from related species.

Component glycerides. The wild-boar fat contains 1·5% of fully saturated glycerides (myristodipalmitin 0·5% and dipalmitostearin 1·0%), 39·6% of disaturated–mono-unsaturated glycerides (dipalmitohexadecenin 3·2%, dipalmito-olein 6·1%, palmitostearohexadecenin 2·0% and

palmitostearo-olein 28·3 %) and 58·9 % of monosaturated–diunsaturated glycerides (hexadeceno-oleopalmitin 25·0 %, hexadeceno-oleostearin 10·7 % and palmitodiolein 23·2 %). No triunsaturated glyceride was found.

The experimental values for the distribution of acyl groups in the glycerides of wild-boar fat, together with the computed values of the 'even' and 'random' types, are given in Table 8, and it is clear that wild-boar fat follows the rule of 'even' distribution, according to which the component fatty acids tend to be distributed as widely as possible among all the triglyceride molecules. In this respect wild-boar fat does not fall into line with the general land-animal type of fat. The 'random' distribution would require an appreciable amount of fully saturated triglycerides, lower proportions of disaturated–mono-unsaturated, and monosaturated–diunsaturated glycerides, and an appreciable quantity of triunsaturated glycerides. As the wild-boar fat follows the 'even-distribution' pattern, Kartha's (1953) theory of restricted random distribution need not be discussed.

SUMMARY

1. The composition of the depot fat from a wild-boar (*Sus cristatus indicus*) has been studied. The mixed acids were separated by applying the lead salt–ethanol method and their composition was studied by the ester-fractionation procedure.

2. The proportions of individual fatty acids in the fat were, in general, similar to those in the fats of other herbivorous animals and were very close to those of pig fat.

3. The glyceride structure of the wild-boar fat has been investigated by low-temperature crystallization of neutral fat with acetone and ether as crystallizing medium. Three main fractions thus obtained were studied separately and possible glyceride composition was computed from the observed data.

4. The observed glyceride pattern in wild-boar fat follows Hilditch's rule of 'even distribution' and so it does not correspond with general land-animal types.

The authors wish to express their thanks to the authorities of the Banaras Hindu University for providing research facilities.

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Plant Enzyme Reactions Leading to the Formation of Heterocyclic Compounds

3. PLANT AMINE OXIDASE AND THE FORMATION OF PYRROLIDINE AND PIPERIDINE ALKALOIDS*

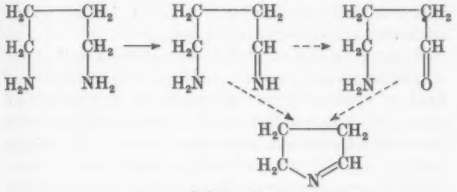
By A. J. CLARKE and P. J. G. MANN
Biochemistry Department, Rothamsted Experimental Station, Harpenden, Herts

(*Received* 29 *August* 1958)

The products of the oxidation of 1:4-diaminobutane and 1:5-diaminopentane, catalysed by plant amine oxidase in the presence of catalase, accumulate in the reaction mixtures as compounds of Δ'-pyrroline and of 2:3:4:5-tetrahydropyridine respectively (Mann & Smithies, 1955*a*; Hasse & Maisack, 1955). Mann & Smithies (1955*a*) suggested that these compounds arise by spontaneous cyclization of the aldimines, which are probably the primary products of the enzyme reactions, or of the amine aldehydes formed from the aldimines by spontaneous reaction with water. Thus with 1:4-diaminobutane as substrate, the suggested reactions were those given in Scheme 1. With 1:4-diaminobutane and 1:5-diaminopentane as enzyme substrates the amine aldehydes postulated as possible intermediates in the reactions would be γ-aminobutyraldehyde and δ-aminovaleraldehyde.

* Part 2: Mann & Smithies (1955*b*).

These aldehydes and their *N*-methyl derivatives, or the cyclic forms of these compounds, have been used as starting materials in model synthesis of alkaloids of the pyrrolidine and piperidine series. Such syntheses depend on the condensation of the



Scheme 1

aldehydes, or their cyclic forms, with β-oxo acids, which undergo decarboxylation during the reactions which result in the formation of β-(2-pyrrolidyl)- or β-(2-piperidyl)-ketones (Anet, Hughes & Ritchie, 1949*a*, *b*; Galinovsky, Wagner & Weiser, 1951; Robinson, 1936; Schöpf, 1949, 1955). The fact that in some cases these condensations occur in aqueous media at pH 7·0 supports the suggestion of Robinson (1917*a*) that such reactions may be involved in the biosynthesis of pyrrolidine and piperidine alkaloids.

The present work records the results of a study of the reactions occurring when the oxidation of a series of aliphatic diamines of varying hydrocarbon chain length, catalysed by plant amine oxidase, was carried out in the presence of certain β-oxo acids. Evidence was obtained that, in some cases, alkaloids were formed as reaction products.

EXPERIMENTAL

Enzyme preparations. Plant amine oxidase preparations were made from the cotyledons of pea seedlings by the method of Clarke & Mann (1957). Fourteen preparations were used during the work. The contents of N varied from 420 to 840 μg./ml., with an average of 590 μg./ml. The specific activities (oxidase units/mg. of N) varied from 60 000 to 156 000, with an average of 94 000. A unit of amine oxidase is defined as the amount which at 28° gives an uptake of 1 μl. of O_2/hr. (calculated from initial rates) in the presence of 0·01 M-1:4-diaminobutane, catalase and 67 mM-phosphate buffer, pH 7·0, in a total volume of 3 ml. (Mann, 1955). Catalase was prepared from ox liver by the method of Sumner & Dounce (1937). The activity of the preparation was estimated by the method of Sumner & Somers (1943). The rate of decomposition of 0·01 N-H_2O_2 in 6·7 mM-phosphate buffer, pH 7·0, at 0°, by a suitable amount of the catalase, was followed by iodine titration of samples taken at 0, 3, 6, 9 and 12 min. From the equation

$$K = \frac{1}{t} \log \frac{a}{a-x},$$

where a is the initial H_2O_2 concentration and $a-x$ is the concentration at time t (min.), K values for the time intervals were calculated and the K value at zero time was obtained by extrapolation. With this value for K the Katalasefähigkeit (Euler & Josephson, 1927; Kat.f. = K/g. of enzyme) was found to be 42 000, suggesting a purity of about 80%. From the equation of Chance & Herbert (1950) that Kat.f. = 520 k_1'/M, where M is the molecular weight of catalase (230 000), the velocity constant of the preparation $k_1' = 1{\cdot}9 \times 10^7\ M^{-1}$ sec.$^{-1}$.

Chemicals. 1:4-Diaminobutane dihydrochloride, 1:5-diaminopentane dihydrochloride, 1:6-diaminohexane and 1:10-diaminodecane were obtained from L. Light and Co. Ltd. Solutions of the free bases were neutralized with 0·2 N-HCl. Acetoacetic acid was prepared by the method of Krueger (1952). Benzoylacetic acid was obtained by hydrolysis of ethyl benzoylacetate, prepared by the method given by Hauser & Hudson (1942). Acetonedicarboxylic acid was prepared by the procedure described by Adams, Chiles & Rassweiler (1941). Pyruvic acid was obtained from British Drug Houses Ltd. and was redistilled immediately before use. Oxaloacetic acid, α-oxoglutaric acid and sodium mesoxalate were obtained from L. Light and Co. Ltd. Solutions of the keto acids were prepared immediately before use and brought to the required pH with 0·2 N-NaOH. Solutions of sodium mesoxalate were brought to the required pH with 0·2 M-KH_2PO_4. *o*-Aminobenzaldehyde was prepared by the method of Bamberger & Demuth (1901, 1927). α-Tripiperideine (perhydro-4a:8a:12a-triazatriphenylene) and *iso*-tripiperideine (perhydro-1:8a:12a-triazatriphenylene) were prepared by the method of Schöpf, Komzak, Braun & Jacobi (1948). Solutions of these compounds were prepared immediately before use and brought to the required pH with 0·2 N-HCl.

Buffers. Phosphate buffers (0·2 M) were prepared from solutions of KH_2PO_4 and KOH.

Paper chromatography. A descending one-dimensional technique was used with Whatman no. 1 paper. The solvent was the upper phase of butan-1-ol–acetic acid–water (4:1:5, by vol.). The detecting agents were 0·1% (w/v) solution of ninhydrin or of isatin or 0·2% (w/v) solution of *o*-aminobenzaldehyde in actone or the modified Dragendorff reagent of Munier & Macheboeuf (1951). With *o*-aminobenzaldehyde and Munier & Macheboeuf's reagent, the colours were developed at room temperature and with ninhydrin and isatin by heating at 100° for 5–10 min.

Manometric methods. Measurements of O_2 uptake were made, in air, in the Warburg apparatus at 28°. The volume of the reaction mixtures was 3 ml., except where otherwise stated. Carbon dioxide was measured by Warburg's direct method (Dixon, 1943). Acetoacetic acid was estimated manometrically by measuring the CO_2 formed in its decarboxylation, catalysed by aniline citrate, according to the method of Edson (1935), except that the reaction was carried out at 28° and the CO_2 evolution was measured over 1 hr.

Melting points. These are uncorrected.

RESULTS

Decarboxylation of β-oxo acids by plant amine oxidase systems

Decarboxylation of acetoacetate. The reactions catalysed by amine oxidases have generally been represented by the equation

$$R{\cdot}CH_2{\cdot}NH_2 + H_2O + O_2 = R{\cdot}CHO + H_2O_2 + NH_3.$$

In the presence of catalase the net reaction is expressed by:

$$2R{\cdot}CH_2{\cdot}NH_2 + O_2 = 2R{\cdot}CHO + 2NH_3.$$

Thus in the oxidation of 1:4-diaminobutane, catalysed by plant amine oxidase in the presence of catalase, the total O_2 uptake was 0·5 mole/mole of amine and no CO_2 was formed. If, however, the oxidation of 1:4-diaminobutane was carried out in the presence of acetoacetate an output of gas took place (Fig. 1). This output was at first comparatively slow, but after a short lag period it reached a rate similar to that of the O_2 uptake. It continued after the oxidation reaction was completed and finally exceeded the O_2 uptake. If either the amine or the amine oxidase was omitted from the reaction mixtures, or if heat-treated enzyme (10 min. at 100°) was used, very little gas output was observed. When the rate of gas output in the complete reaction mixture had fallen to a low level, the amount of acetoacetate present was estimated by decarboxylation with aniline citrate. The CO_2 evolved in this estimation was less than that required for the acetoacetate originally present, by an amount approximately equal to the gas output during the oxidation reaction. This gas was CO_2, produced by decarboxylation of the acetoacetate. Only traces of carbonyl compounds, steam-volatile at pH 5·2, were found in the complete reaction mixture. The reaction therefore differs from the known decarboxylation of β-oxo acids by primary amines, which yields acetone from acetoacetic acid. The fact that such CO_2 evolution was dependent on the presence of both the amine and the amine oxidase suggested that it was due to a reaction between an oxidation product of the amine and the acetoacetate and not to the amine acting as a decarboxylating agent.

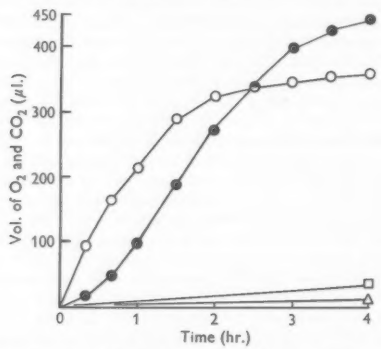


Fig. 1. Uptake of O_2 and output of CO_2 during the amine oxidase-catalysed oxidation of 1:4-diaminobutane in the presence of acetoacetate. Reaction mixtures, of 3 ml. total volume, consisted of amine oxidase (0·02 ml.), catalase (50 μg.) and acetoacetate (30 μmoles) in 33 mM-phosphate, pH 5·2. The substrate (30 μmoles of 1:4-diaminobutane) was added from the side arm. The amine and the amine oxidase respectively were omitted from control-reaction mixtures. Gas phase, air; temp., 28°. Uptake of O_2: ○, complete reaction mixture. Output of CO_2: ●, complete reaction mixture; □, amine oxidase omitted; △, 1:4-diaminobutane omitted.

Effect of variation of diamine substrate and of pH *on the decarboxylation of acetoacetate by plant amine oxidase systems.* The oxidation of a number of diamines, catalysed by plant amine oxidase in the presence of catalase, was carried out in phosphate buffers, pH 5·2, 8·0 and 8·5, in reaction mixtures containing acetoacetate at the same concentration as that of the diamines (0·01 M). The reaction mixtures were incubated for 5 hr., by which time the O_2 uptakes had reached, or exceeded, the theoretical values, and the evolution of CO_2 (which lagged behind the O_2 uptakes, as in the experiment of Fig. 1) had fallen to a very low rate. Samples (1 ml.) of the reaction mixtures were then transferred to Warburg vessels with two side arms, and bound CO_2 was measured over a 10 min. period after the addition of 0·3 ml. of 50% (w/v) citric acid. Residual acetoacetate was then estimated in the same samples by measuring the CO_2 output in 1 hr., after the addition of aniline citrate (0·4 ml. of aniline–50% citric acid, 1:1) from the second side arm. The values given in Table 1 for total CO_2 and decrease in acetoacetate are corrected for the spontaneous decomposition of acetoacetate in control-reaction mixtures from which the amine oxidase was omitted; the CO_2 formed did not exceed 2 μmoles. The O_2 uptakes in the reaction mixtures at pH 7 and 8·5 were generally higher than the theoretical values. The causes of such increased O_2 uptakes, with 1:5-diaminopentane and 1:10-diaminodecane as substrates, have been investigated by Mann & Smithies (1955*a*). With 1:5-diaminopentane it was attributed to the further oxidation, catalysed by Mn^{2+} ions and peroxidase, of the ring compounds formed by the cyclization of the primary reaction product. With 1:10-diaminodecane, the increased O_2 uptakes have been shown to be due to the slow oxidation of the amine aldehyde, which is the product of the primary reaction.

The total CO_2 (free and bound) varied with the amine used as substrate for the oxidase. It was greatest with 1:4-diaminobutane, where it exceeded the O_2 uptake, and was least with 1:10-diaminodecane. Changes in pH, from 5·2 to 8·5, did not alter the amount of CO_2 formed. The CO_2 outputs were accompanied by corresponding decreases in the concentration of acetoacetate.

Effect of variation of keto acid. The oxidation of 1:4-diaminobutane, catalysed by the amine oxidase in the presence of a number of different keto acids,

and the CO_2 outputs, were measured (Table 2). The reaction mixtures were buffered with phosphate, pH 5·5, and no correction was made for bound CO_2. The experiments were designed to test the relative rates of decarboxylation of the different keto acids by the system. The evolution of CO_2 was measured over a period of 1 hr., for the amount of enzyme preparation used was such that the O_2 uptakes approached the theoretical value in this time. The results show that, of the acids tested, only the β-oxo acids were decarboxylated by the amine oxidase system. The rates of decarboxylation of benzoylacetic acid and of acetonedicarboxylic acid were much greater than that of acetoacetic acid. The effect of the amine oxidase system on the decarboxylation of oxaloacetic acid was not clearly shown under the conditions of Table 2, owing to the comparatively rapid spontaneous decarboxylation of this acid and the marked effect of 1:4-diaminobutane alone on the rate of decarboxylation. The fact that the amine oxidase system is much more active in the reaction than the amine alone is shown more clearly by the experiments of Fig. 2, in which lower concentrations of oxaloacetate and 1:4-diaminobutane were used to reduce the rates of CO_2 output in the control-reaction mixtures.

Effect of variation of diamine substrate on the decarboxylation of benzoylacetate. The decarboxylation of benzoylacetate by amine oxidase systems was tested in reaction mixtures, with different diamine substrates, in phosphate buffer, pH 5·2. The O_2 uptakes and CO_2 outputs of the complete reaction mixtures and of control-reaction mixtures, from which either the amine oxidase or the amine was omitted, were measured over 4 hr. The results (Table 3) show that the CO_2 formed was much higher than in similar experiments with acetoacetate (Table 1) and, with 1:4-diaminobutane as

Table 1. *Effect of variation of* pH *and of diamine substrate on the decarboxylation of acetoacetate by amine oxidase systems*

Reaction mixtures, of 3 ml. total volume, consisted of amine oxidase, catalase (50 μg.) and acetoacetate (30 μmoles) in 33 mM-phosphate. Diamine substrates (30 μmoles) were added from the side arms. Amine oxidase was omitted from the control vessels. The O_2 uptakes and CO_2 outputs were measured for 5 hr. at 28° in air. Bound CO_2 and residual acetoacetate were then estimated on samples of the reaction mixtures. Values for CO_2 formed and decrease in acetoacetate are corrected for the spontaneous decomposition of acetoacetate in the control-reaction mixtures.

Amine	Amine oxidase (ml.)	Phosphate (pH)	Uptake of O_2 (μmoles)	Output of CO_2 (μmoles)	Decrease in acetoacetate (μmoles)
1:4-Diaminobutane	0·02	5·2	14·6	15·6	17·6
1:4-Diaminobutane	0·01	7·0	15·0	17·6	19·0
1:4-Diaminobutane	0·02	8·5	15·6	15·8	12·7
1:5-Diaminopentane	0·02	5·2	14·5	6·6	8·1
1:5-Diaminopentane	0·01	7·0	16·2	5·2	5·4
1:5-Diaminopentane	0·02	8·5	15·8	8·9	8·6
1:6-Diaminohexane	0·1	5·2	15·5	14·5	13·7
1:6-Diaminohexane	0·01	7·0	18·9	13·1	15·3
1:6-Diaminohexane	0·005	8·5	18·4	13·7	14·0
1:10-Diaminodecane	0·1	5·2	14·1	1·1	1·1
1:10-Diaminodecane	0·02	7·0	21·2	1·7	2·4
1:10-Diaminodecane	0·02	8·5	16·5	0·6	1·0

Table 2. *Decarboxylation of keto acids by amine oxidase systems*

Reaction mixtures, of 3 ml. total volume, consisted of amine oxidase (0·02 ml.), catalase (50 μg.) and the sodium salts of the keto acids (30 μmoles) in 33 mM-phosphate, pH 5·5. Substrate (30 μmoles of 1:4-diaminobutane) was added from the side arm. Amine oxidase and 1:4-diaminobutane respectively were omitted from control-reaction mixtures. The O_2 uptakes and CO_2 outputs were measured over 1 hr. Other conditions were similar to those described in Fig. 1.

Keto acid (as sodium salt)	Uptake of O_2 (μl./hr.) by complete reaction mixture	Output of CO_2 (μl./hr.)		
		1:4-Diaminobutane omitted	Amine oxidase omitted	Complete reaction mixture
Pyruvic acid	301	2	4	2
Acetoacetic acid	308	8	13	114
Benzoylacetic acid	291	7	4	307
Mesoxalic acid	310	7	0	2
Oxaloacetic acid	314	73	275	474
α-Oxoglutaric acid	323	2	2	21
Acetonedicarboxylic acid	282	5	27	466

substrate, approached 1 mole of CO_2/mole of amine. With 1:10-diaminodecane as substrate, a slight white turbidity was noted at the end of the experiment. This was not observed in the control-reaction mixtures, nor in the complete reaction mixtures with the other diamines as substrates.

Decarboxylation of β-oxo acids by the products of the oxidation of the diamines

Evidence that the observed decarboxylation of acetoacetate is brought about by the products of the oxidation of the diamines was obtained in experiments in which the enzymic oxidation of the diamines was taken to completion (0·5 mole of O_2/mole of amine) before the addition of acetoacetate. Fig. 3 shows the subsequent CO_2 outputs in one such experiment, with 10, 20 and 30 μmoles of 1:4-diaminobutane as substrate in phosphate buffer, pH 5·2. Similar CO_2 outputs were observed

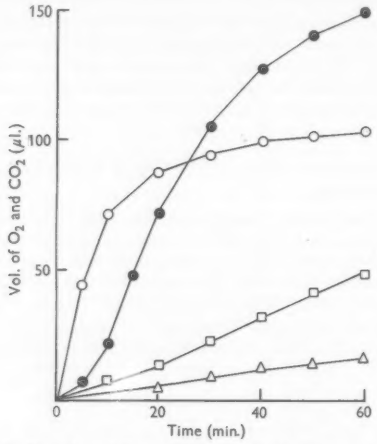


Fig. 2. Decarboxylation of oxaloacetate by amine oxidase systems. Reaction mixtures consisted of amine oxidase (0·02 ml.), catalase (50 μg.) and oxaloacetate (10 μmoles) in 33 mM-phosphate, pH 5·5. The substrate (10 μmoles of 1:4-diaminobutane) was added from the side arm. The amine and the amine oxidase respectively were omitted from control-reaction mixtures. Other conditions were similar to those in Fig. 1. Uptake of O_2: ○, complete reaction mixture. Output of CO_2: ●, complete reaction mixture; □, amine oxidase omitted, △, 1:4-diaminobutane omitted.

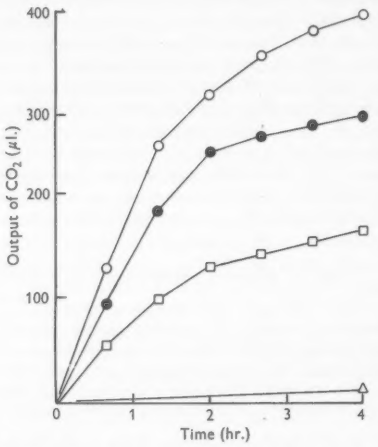


Fig. 3. Decarboxylation of acetoacetate by the products of the amine oxidase-catalysed oxidation of 1:4-diaminobutane. Reaction mixtures, of 3 ml. total volume, consisted of amine oxidase (0·05 ml.) and catalase (50 μg.) in 33 mM-phosphate, pH 5·2. The substrate (10, 20 and 30 μmoles of 1:4-diaminobutane) was added from the side arm. In a control-reaction mixture the amine was omitted. Incubation was continued until the O_2 uptake stopped (0·5 mole of O_2/mole of amine). Acetoacetate (30 μmoles) was then added to the complete and control-reaction mixtures and the subsequent outputs of CO_2 were measured. Other conditions were similar to those of Fig. 1. Output of CO_2: ○, 30 μmoles of 1:4-diaminobutane; ●, 20 μmoles of 1:4-diaminobutane; □, 10 μmoles of 1:4-diaminobutane; △, control-reaction mixture without 1:4-diaminobutane.

Table 3. *Decarboxylation of benzoylacetate by amine oxidase systems*

Reaction mixtures, of 3 ml. total volume, consisted of amine oxidase, catalase (50 μg.) and benzoylacetate (30 μmoles) in 33 mM-phosphate, pH 5·2. Diamine substrates (30 μmoles) were added from the side arms. Amine and the amine oxidase respectively were omitted from control-reaction mixtures. The O_2 uptakes and CO_2 outputs were measured over 4 hr. Other conditions were similar to those of Fig. 1.

Amine	Amine oxidase (ml.)	Uptake of O_2 (μmoles) by complete reaction mixture	Output of CO_2 (μmoles)		
			Amine omitted	Amine oxidase omitted	Complete reaction mixture
1:4-Diaminobutane	0·02	15·9	2·0	1·8	25·7
1:5-Diaminopentane	0·02	14·6	1·8	1·9	13·3
1:6-Diaminohexane	0·1	17·6	1·8	1·8	21·7
1:10-Diaminodecane	0·1	14·1	1·7	2·1	7·0

when the amine oxidase-reaction mixtures were filtered, in an ultrafiltration apparatus of the type described by Paterson, Pirie & Stableforth (1947), before addition of the acetoacetate, showing that once the oxidation of the diamine was complete the subsequent decarboxylation reaction was independent of the presence of the enzyme. In contrast with the results of Fig. 1, where the acetoacetate was present during the oxidation, there was little or no lag period before the CO_2 output started. Although the evolution of CO_2 was not complete in the 4 hr. experimental period, particularly with the higher concentrations of 1:4-diaminobutane, the results suggest a stoicheiometric relation between the amount of amine oxidized and the amount of acetoacetate decarboxylated. The CO_2 evolution was most nearly complete with the lowest amount of amine, where it reached about 160 μl. (theoretical amount for mole/mole reaction 224 μl. of CO_2). The stoicheiometric relation between the amount of 1:4-diaminobutane oxidized and the CO_2 output was shown more clearly in similar experiments in which acetonedicarboxylate was used instead of acetoacetate (Fig. 4). In these experiments, the amounts of 1:4-diaminobutane used were 5, 10 and 15 μmoles. The CO_2 outputs were complete within the experimental period and were only slightly less than 1 mole of CO_2/mole of 1:4-diaminobutane oxidized. The results also show that acetonedicarboxylate is rapidly decarboxylated by low concentrations of the oxidation product of 1:4-diaminobutane.

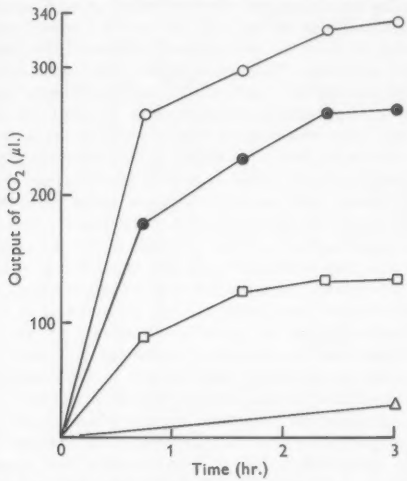


Fig. 4. Decarboxylation of acetonedicarboxylate by the products of the amine oxidase-catalysed oxidation of 1:4-diaminobutane. Conditions were as in Fig. 3, except that acetonedicarboxylate (30μmoles) was substituted for acetoacetate and the amounts of 1:4-diaminobutane used were lower. Outputs of CO_2: ○, 15 μmoles of 1:4-diaminobutane; ●, 10 μmoles of 1:4-diaminobutane; □, 5 μmoles of 1:4-diaminobutane; △, control-reaction mixture without 1:4-diaminobutane.

Effect of variation of pH *and of diamine substrate on the decarboxylation of acetoacetate by the products of amine oxidase reactions.* The effects of variation of pH and of diamine substrate on the decarboxylation of acetoacetate by the products of the amine oxidase reactions were also studied (Table 4). The oxidations were carried out in phosphate buffers pH 5·2, 7·0 and 8·5, with comparatively large

Table 4. *Decarboxylation of acetoacetate by the products of the amine oxidase-catalysed oxidation of various diamines*

Reaction mixtures, of 3 ml. total volume, consisted of amine oxidase and catalase (50 μg.) in 33 mM-phosphate. Diamine substrates (30 μmoles) were added from the side arms. Reaction mixtures were incubated, in air, until the O_2 uptakes reached about 0·5 mole/mole of substrate. The vessels were then filled with N_2 and acetoacetate (30 μmoles) was added from the side arms. Subsequent CO_2 outputs were measured over 4 hr. Bound CO_2 was then measured over 10 min., after the addition of 0·3 ml. of 50% (w/v) citric acid from the side arm. The values given for total CO_2 are corrected for those obtained in control-reaction mixtures with acetoacetate but without the diamines.

Amine	Amine oxidase (ml.)	Phosphate (pH)	Oxidation time (min.)	Uptake of O_2 (μmoles)	Total output of CO_2 (μmoles)
1:4-Diaminobutane	0·04	5·2	60	14·3	20·2
1:4-Diaminobutane	0·02	7·0	35	15·6	12·7
1:4-Diaminobutane	0·04	8·5	45	14·6	13·3
1:5-Diaminopentane	0·04	5·2	60	13·7	8·7
1:5-Diaminopentane	0·03	7·0	50	14·1	2·4
1:5-Diaminopentane	0·04	8·5	40	14·5	1·1
1:6-Diaminohexane	0·3	5·2	66	14·1	13·2
1:6-Diaminohexane	0·03	7·0	66	13·9	11·2
1:6-Diaminohexane	0·02	8·5	55	15·0	9·1
1:10-Diaminodecane	0·3	5·2	40	13·9	0·7
1:10-Diaminodecane	0·04	7·0	50	15·4	0·3
1:10-Diaminodecane	0·03	8·5	45	15·2	0·6

amounts of the enzyme preparation, so that the theoretical O_2 uptakes were reached within 1 hr. Under these conditions, the secondary oxidations already noted in connexion with the experiments of Table 1 became more marked, and for this reason the subsequent reactions with acetoacetate were carried out in an atmosphere of N_2. The CO_2 outputs were measured over a period of 4 hr. after addition of the acetoacetate. The results showed that, with 1:4-diaminobutane and 1:6-diaminohexane as substrates, the CO_2 formed was not much less than that when the acetoacetate was present during the oxidation of the amines, and decreased with rise in pH (Table 1). However, with 1:5-diaminopentane at pH 7·0 and 8·5 the CO_2 outputs were much smaller than those of Table 1 and with 1:10-diaminodecane no significant amounts of CO_2 were formed. This connexion between CO_2 output and pH suggested that the oxidation products causing the decarboxylation were most stable at pH 5·2, and that the oxidation product formed from 1:5-diaminopentane was less stable than those from 1:4-diaminobutane and 1:6-diaminohexane.

Decarboxylation of acetoacetate and acetonedicarboxylate by α- and iso*-tripiperideine.* The unsaturated ring compound resulting from the amine oxidase-catalysed oxidation of 1:5-diaminopentane is 2:3:4:5-tetrahydropyridine. The work of Schöpf and his collaborators (e.g. Schöpf *et al.* 1948; Schöpf, Braun & Komzak, 1956) has shown that this compound is only stable in strongly acid solutions. In weakly acid, neutral or alkaline solutions it polymerizes to the dimer, tetrahydroanabasine or to the two trimers, α- and *iso*-tripiperideine. The effect of these two trimers on the decarboxylation of acetoacetate and acetonedicarboxylate was tested in phosphate buffers, pH 5·2, 7·0 and 8·5. The reaction mixtures were incubated in N_2 and the total CO_2 (free and bound) formed in 4 hr. was measured. The results (Table 5) show the rates of decarboxylation of acetoacetate and acetonedicarboxylate by both trimers was slow. Similar results were obtained when solutions of α-tripiperideine buffered at pH 5·2, 7·0 and 8·5 were incubated for 5 hr. at 28°, before addition of the keto acid solutions. This preliminary treatment converts the greater part of the α-tripiperideine into the dimer, tetrahydroanabasine (Schöpf *et al.* 1956). The volumes of CO_2 evolved from acetoacetate were less than those in the corresponding experiments of Table 1, where acetoacetate was incubated with amine oxidase-reaction mixtures containing 1:5-diaminopentane as substrate, but of the same order as those of Table 4, where acetoacetate was incubated with the oxidation product of 1:5-diaminopentane. The results suggest that, at least in part, the comparatively low activity in the decarboxylation of β-oxo acids shown by amine oxidase-reaction mixtures containing 1:5-diaminopentane as substrate may be due to the tendency of the reactive cyclic form of the oxidation product to polymerize. Another factor which may be responsible for the comparatively low activity in the decarboxylation of β-oxo acids by amine oxidase systems with 1:5-diaminopentane as substrate, particularly under the conditions of Table 4, where the diamine was oxidized before addition of the acetoacetate, is the secondary oxidation which takes place so readily with this amine as the substrate (Mann & Smithies, 1955*a*).

Table 5. *Decarboxylation of acetoacetate and acetonedicarboxylate by α- and* iso*-tripiperideine*

Reaction mixtures, of 3 ml. total volume, consisted of 10 μmoles of α- or *iso*-tripiperideine in 33 mM-phosphate, pH 5·2, 7·0 and 8·5. Acetoacetate or acetonedicarboxylate (30 μmoles) was added from the side arm. The CO_2 outputs were measured over 4 hr. Bound CO_2 was then measured during 10 min., corrected for values obtained with control-reaction mixtures without α- or *iso*-tripiperideine. Gas phase, N_2; temp. 28°.

Amine	Phosphate (pH)	Total CO_2 (μmoles) Aceto-acetate	Acetone-dicarboxylate
α-Tripiperideine	5·2	4·3	14·1
α-Tripiperideine	7·0	2·2	7·7
α-Tripiperideine	8·5	1·6	9·9
iso-Tripiperideine	5·2	2·5	10·7
iso-Tripiperideine	7·0	1·9	8·6
iso-Tripiperideine	8·5	2·4	11·3

Chromatographic examination of the reaction products

The oxidation of each of the four amines, catalysed by plant amine oxidase in the presence of catalase, was carried out in the presence and in the absence of acetoacetate or benzoylacetate, respectively, under conditions similar to those of Table 2 except that the buffer used was 0·02M-phosphate, pH 5·2. After incubation for 5 hr. the reaction mixtures were evaporated to dryness *in vacuo*. The residues were each suspended in 0·2 ml. of ethanol, and 0·005 ml. samples of the supernatants were chromatographed on Whatman no. 1 paper with the butan-1-ol–acetic acid–water (4:1:5, by vol.) solvent mixture. The results obtained with the various detecting agents are summarized in Table 6. Only the most clearly defined spots are recorded. It was shown by Schöpf & Oechler (1936) and Schöpf *et al.* (1948) that compounds of Δ′-pyrroline and of 2:3:4:5-tetrahydropyridine give yellow and orange colours respectively with *o*-aminobenzaldehyde, owing to the formation of dihydroquinazolinium compounds. With this detecting agent on the paper

Table 6. *Paper chromatography of the products of the oxidation of various diamines in the presence and the absence of acetoacetate or benzoylacetate*

Reaction mixtures, of 3 ml. total volume, consisted of amine oxidase, catalase (50 μg.) and diamine (30 μmoles) with and without acetoacetate or benzoylacetate (30 μmoles) in 0·02 M-phosphate, pH 5·2. Amounts of amine oxidase used varied from 0·02 to 0·10 ml., with the different diamines, as in Table 1. KOH was omitted from the centre wells. Other conditions were similar to those described in Table 1. Incubations were continued for 5 hr. and reaction products were then investigated by paper chromatography as described in the text.

Detecting agents		*o*-Aminobenzaldehyde		Ninhydrin		Isatin		Munier & Macheboeuf's (1951) reagent	
Amine	Keto acid	Colour	Mean R_F	Colour	Mean R_F	Colour	Mean R_F	Colour	Mean R_F
1:4-Diaminobutane	None	Yellow	0·18	Yellow–brown	0·17	None	—	Pink	0·19
1:4-Diaminobutane	Acetoacetic	None	—	Yellow	0·47	Dark blue	0·47	Pink	0·48
1:4-Diaminobutane	Benzoylacetic	None	—	Yellow	0·72	Dark blue	0·72	Pink	0·72
1:5-Diaminopentane	None	Orange	0·27	Purple–brown	0·27	None	—	Pink	0·27
1:5-Diaminopentane	Acetoacetic	None	—	Purple with yellow centre	0·56	Pale blue	0·57	Pink	0·56
1:5-Diaminopentane	Benzoylacetic	None	—	Purple with yellow centre	0·80	Pale blue	0·78	Pink	0·80
1:6-Diaminohexane	None	Weak yellow	0·34	Purple–slate	0·34	None	—	None	—
1:6-Diaminohexane	Acetoacetic	None	—	Weak purple	0·63	Pale blue	0·65	Pink	0·64
1:6-Diaminohexane	Benzoylacetic	None	—	Purple with yellow centre	0·79	Pale blue	0·79	Pink	0·81
1:10-Diaminodecane	None	Weak yellow	0·82	Purple	0·66	None	—	Pink	0·65
				Purple	0·80	—	—	—	—
1:10-Diaminodecane	Acetoacetic	None	—	Purple	0·66	None	—	Pink	0·70
1:10-Diaminodecane	Benzoylacetic	None	—	Purple	0·65	None	—	Pink	0·63
				Purple	0·87	—	—	Pink	0·86

chromatograms, strong yellow or orange spots respectively were given by the reaction mixtures containing 1:4-diaminobutane and 1:5-diaminopentane as substrates, in the absence of the keto acids. With 1:6-diaminohexane and 1:10-diaminodecane as substrates, in the absence of keto acids, yellow spots were obtained with the reagent, but these were weak, particularly where 1:10-diaminodecane was the substrate. The compounds detected with *o*-aminobenzaldehyde gave weak or negative colour reactions with the other detecting agents. In the complete reaction mixtures containing acetoacetate or benzoylacetate in addition to the amine substrates, the compounds reacting with *o*-aminobenzaldehyde were not detected, suggesting that the reactions with these keto acids involved the disappearance of the unsaturated ring compounds. In such reaction mixtures, however, different products appeared which were detected with ninhydrin and Munier & Macheboeuf's reagent and, except in the 1:10:diaminodecane reaction mixtures, with isatin. In such reaction mixtures with 1:4-diaminobutane and 1:5-diaminopentane as substrates, the colours obtained with ninhydrin and isatin resembled those given by pyrrolidine and piperidine respectively, suggesting that the reactions with the β-oxo acids resulted in the formation of saturated ring compounds.

Isolation of reaction products

General procedure. Reaction mixtures, in 250 ml. Erlenmeyer flasks, each consisted of 1 ml. of amine oxidase, 0·5 mg. of catalase, 20 ml. of 0·1 M-diamine substrate (1:4-diaminobutane or 1:5-diaminopentane), 24 ml. of 0·1 M-acetoacetate and 6 ml. of 0·2 M-phosphate, pH 6·0, in a total volume of 60 ml. Four such reaction mixtures were used with each substrate. The flasks were shaken in the bath at 28° for 5 hr. To remove residual acetoacetate, the combined reaction mixtures and washings were passed through a column (50 cm. × 1·4 cm.) of De-Acidite FF (−16+50 mesh; The Permutit Co. Ltd., London, W. 4) in the hydroxide form, and washed through with 100 ml. of water. As the solution emerged from the column it was brought to pH 6·0 by dropwise addition of 2 N-H_2SO_4. The solution was cooled in ice, made strongly alkaline by adding 2·5 g. of KOH in 20 ml. of water and continuously extracted with ether for 48 hr. in an apparatus swept with N_2. Evaporation of the ether *in vacuo* left the free base as a colourless or pale-yellow liquid which rapidly darkened and resinified on exposure to air. In general it was more convenient to isolate the bases in the form of their hydrochlorides. A slight excess of an ethereal solution of hydrogen chloride was added immediately to the ether extract. After removal of the ether under reduced pressure and drying in a vacuum desiccator, a resinous product was obtained which slowly crystallized on standing. The product was extracted with successive portions of hot, dry ethyl acetate. On concentration and cooling of the combined ethyl acetate extracts, colourless crystals of the base hydrochloride were obtained.

(±)-2-*Pyrrolidylacetone* (*norhygrine*). The norhygrine hydrochloride (0·44 g.; 34 % yield based on 80 ml. of 0·1 M-1:4-diaminobutane) crystallized in needles from dry ethyl acetate. The deliquescent crystals had m.p. 114–115° (Found: N, 8·3. Calc. for $C_7H_{13}ON,HCl$: N, 8·6 %). Chromatographed on Whatman no. 1 paper with the solvent system butan-1-ol–acetic acid–water (4:1:5, by vol.), and isatin as detecting agent, the compound gave one dark-blue spot, R_F 0·47 (see Table 5). Both the free base and the hydrochloride gave positive iodoform tests. Attempts to prepare the picrate of the base gave an orange–red gum which could not be crystallized.

The hydrochloride, or the free base, readily yielded a semicarbazone. A solution of the base (62 mg.) in pyridine (1 ml.) was treated with a slight excess of semicarbazide hydrochloride in aqueous pyridine (0·5 ml.; 1:1, v/v). The mixture was then heated on a water bath for 1 hr. On cooling the reaction mixture a fine white powder separated which was filtered off and dried (53 mg.; m.p. 193–194°). Recrystallization from a small volume of methanol gave a colourless crystalline product, m.p. 195–196°, which after drying *in vacuo* over phosphorus pentoxide had m.p. 204–205° (Found: C, 40·6; H, 8·3; N, 23·1. Calc. for $C_8H_{16}ON_4,H_2O,HCl$: C, 40·3; H, 8·0; N, 23·5 %).

A solution of platinic chloride in 2 N-HCl was added to an aqueous solution of the base hydrochloride and the mixture allowed to stand for 30 min. The precipitate which formed was filtered off and washed thoroughly with water and then with ethanol. The buff-coloured precipitate decomposed in hot water and was insoluble in organic solvents. Without further purification the product was dried *in vacuo*. The product had m.p. 228° (decomp.) [Found: C, 25·6; H, 4·5; N, 3·9. Calc. for $(C_7H_{13}ON)_2,H_2PtCl_4$: C, 25·4; H, 4·2; N, 4·2 %]. The residue after ignition was 28·8 % of the initial weight, giving a molecular weight of 135 for a mono-acid base. The molecular weight expected for norhygrine is 127.

Norhygrine and its derivatives are not recorded in the literature, though C. Schöpf (personal communication, 1956) has prepared norhygrine by condensation of Δ′-pyrroline with acetoacetate.

(±)-2-*Piperidylacetone* (*iso*pelletierine). The base was isolated in the manner previously described, and on treatment with an ethereal solution of hydrogen chloride yielded the crude *iso*pelletierine

hydrochloride. The base hydrochloride was extracted with portions of hot, dry ethyl acetate and the extracts were concentrated. On cooling the resulting solution, white flocculent crystals of the hydrochloride were obtained, m.p. 137–140°, raised to 141–143° on recrystallization from ethyl acetate (0·14 g.; 12% yield) (Found: C, 54·05; H, 8·3; N, 8·1; Cl, 20·2. Calc. for $C_8H_{16}ONCl$: C, 54·1; H, 8·4; N, 7·9; Cl, 20·0%). Mortimer & Wilkinson (1957) give m.p. 144–145°. Chromatographed on Whatman no. 1 paper with the solvent system butan-1-ol–acetic acid–water (4:1:5, by vol.), and isatin as detecting agent, the compound gave one pale-blue spot R_F 0·57.

A sample of the base hydrochloride was used to prepare the semicarbazone derivative in a manner similar to that given for norhygrine hydrochloride. The semicarbazone was obtained as a white powder, m.p. 158–160°, after recrystallization from methanol, m.p. 167–168° (Found: C, 42·9; H, 8·1; N, 21·9. Calc. for $C_9H_{18}O_4N,H_2O,HCl$: C, 42·8; H, 8·4; N, 22·2%). Mortimer & Wilkinson (1957) give m.p. 169–171°. An authentic sample kindly supplied by Dr Wilkinson had m.p. 169–170° unchanged by admixture with the semicarbazone described above.

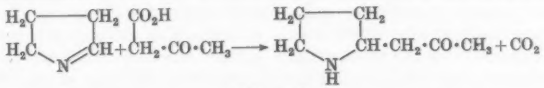
The picrate was obtained by treating a solution of the free base in ethanol with an ethanolic solution containing the calculated amount of picric acid. The picrate crystallized in feathery clusters of needles and, after recrystallization from ethanol, had m.p. 147–148°. Mortimer & Wilkinson (1957) give m.p. 149–150°. An authentic sample kindly supplied by Dr Wilkinson had m.p. 147–148° unchanged by admixture with the sample described above (Found: C, 45·55; H, 5·0; N, 15·4. Calc. for $C_8H_{15}ON,C_6H_3O_7N_3$: C, 45·4; H, 5·0; N, 15·1%). Determinations of the molecular weight of the picrate by the method of Cunningham, Dawson & Spring (1951) gave values of 361 and 358. The value expected for the picrate of *iso*pelletierine is 370.

DISCUSSION

The results of the present work suggest that the observed decarboxylation of β-oxo acids by the amine oxidase systems used is brought about by stoicheiometric reactions between the products of the amine oxidase reactions and the β-oxo acids. Of the diamines used as substrates 1:4-diaminobutane, 1:5-diaminopentane and 1:6-diaminohexane were active in the system, whereas 1:10-diaminodecane was comparatively inactive. It is already known (Hasse & Maisack, 1955; Mann & Smithies, 1955*a*) that the amine aldehydes which are the probable products of the amine oxidase-catalysed oxidation of 1:4-diaminobutane and 1:5-diaminopentane spontaneously cyclize to give unsaturated pyrrolidine or piperidine compounds. Preliminary investigations of the oxidation product of 1:6-diaminohexane suggest that it exists under the experimental conditions as an equilibrium mixture of the open chain and cyclic forms. Evidence that the oxidation product of 1:10-diaminodecane reacts primarily as an amine aldehyde was given by Mann & Smithies (1955*a*). The present results therefore support the suggestion of van Tamelen & Knapp (1955), for such reactions with δ-aminovaleraldehyde, that the unsaturated cyclic forms of the amine aldehydes are the reactive species. Thus the reaction between the oxidation products of 1:4-diaminobutane and acetoacetic acid to form norhygrine may be represented as in Scheme 2.

van Tamelen & Knapp (1955) suggested that the reaction involves a cyclic intermediate from which carbon dioxide is eliminated simultaneously with the condensation. An intermediate of this type provides a pathway of decarboxylation differing from that suggested by Pederson (1934) and Westheimer & Jones (1941) for the primary amine-catalysed decarboxylation of β-oxo acids. The slight decarboxylating activity of amine oxidase systems with 1:10-diaminodecane as substrate may be due to the presence of traces of the cyclic form of the oxidation product of this amine. This is suggested by the weakly positive colour test with *o*-aminobenzaldehyde given by reaction mixtures containing this substrate in the absence, but not in the presence, of β-oxo acids. Preliminary results suggest that, contrary to the results of Mann (1955), the oxidation of ornithine is slowly catalysed by the amine oxidase with the formation of a pyrroline carboxylic acid, and that this compound and the tetrahydropicolinic acid formed by the oxidation of lysine also condense with β-oxo acids.

Mann & Smithies (1955*a*) suggested that the oxidation of 1:4-diaminobutane, 1:5-diaminopentane and lysine, catalysed by plant amine oxidase, might be a stage in the biosynthesis of pyrrolidine and piperidine compounds in higher plants. The reactions studied in the present work represent one possible mechanism of such biosynthesis and are of particular interest since the products are alkaloids.



Scheme 2

The results lend additional support to the theory of the biogenesis of such alkaloids advanced by Robinson (1917*a*). In connexion with the present work the following postulate put forward by Robinson (1949) in discussing the synthesis of alkaloids by condensation of amine aldehydes and β-oxo acids is of particular interest: 'Now suppose an aldehyde is produced by an oxidizing enzyme system, is it not possible that acetoacetic, or some other activated acetone derivative, is present in any case and that the reaction will go to completion.'

Unsaturated pyrrolidine and piperidine compounds are formed as a result of reactions catalysed by enzymes other than amine oxidases. Glutamic semialdehyde, which is presumably the primary product of the oxidation of ornithine by the amine oxidase, is an intermediate in the interconversion of glutamic acid, proline and ornithine in animal tissues (Stetten, 1951) and in *Neurospora* (Vogel & Bonner, 1954), and is a precursor of proline in *Escherichia coli* (Vogel & Davis, 1952). It may arise by oxidation of proline (Taggart & Krakaur, 1949), by reduction of glutamic acid (Vogel & Bonner, 1954), by the action of ornithine transaminase (Fincham, 1953; Meister, 1954*a*) or by oxidation of α-amino-δ-hydroxy-valeric acid (Yura & Vogel, 1957). The position in intermediary metabolism of the corresponding aldehyde derived from lysine which spontaneously cyclizes, probably to 2:3:4:5-tetrahydropicolinic acid, is not yet clear. It is a postulated precursor of lysine (Schweet, Holden & Lowy, 1954; Yura & Vogel, 1957) and Mann & Smithies (1955*a*) suggest that it may be a precursor of pipecolinic acid in higher plants. In animal tissues pipecolinic acid is formed from lysine by way of the corresponding α-oxo acid (Rothstein & Miller, 1954). The only enzyme, other than plant amine oxidase, which has so far been shown to catalyse the formation of lysine semialdehyde appears to be the ω-hydroxy-α-amino acid dehydrogenase of Yura & Vogel (1957). The α-oxo acids formed by the action of amino acid oxidases on ornithine and lysine cyclize spontaneously to Δ′-pyrroline-2-carboxylic acid and 3:4:5:6-tetrahydropicolinic acid (Boulanger & Osteux, 1952; Meister, 1954*b*; Boulanger, Coursaget, Bertrand & Osteux, 1957). It is probable that these unsaturated ring compounds, like those derived from the amine aldehydes, would undergo spontaneous condensation reactions with β-oxo acids. It is clear from these considerations that if biosynthesis of pyrrolidine and piperidine alkaloids in higher plants occurs by the type of reaction studied in the present work it is not necessarily dependent on the presence of plant amine oxidase.

With the diamines as substrates the products of the condensation reactions would be racemic mixtures and acetonedicarboxylic, benzoylacetic and propionylacetic acids would, as with acetoacetic acid, result in the formation of naturally occurring α-substituted pyrrolidine and piperidine alkaloids, from which other alkaloids might be formed subsequently by *N*-methylation or by reduction of the carbonyl group. Since one metabolic pathway of proline metabolism involves its oxidation to glutamic semialdehyde, which is in equilibrium with Δ′-pyrroline-5-carboxylic acid, it is possible that $\alpha\alpha_1$-disubstituted pyrrolidine and piperidine alkaloids might be formed by oxidation of the α-substituted alkaloids followed by condensation reactions. Such a possibility has been mentioned in connexion with alkaloids of *Lobelia inflata* (Schöpf & Kauffman, 1957). Where acetonedicarboxylic acid, or a half-ester of this acid, has taken part in the initial condensation, further condensation subsequent to oxidation of the ring of the monosubstituted pyrrolidine or piperidine compound could give rise to alkaloids having a tropane or ψ-pelletierine structure. This suggests a biosynthetic pathway alternative to the succinic dialdehyde synthesis of tropinone (Robinson, 1917*b*; Schöpf & Lehmann, 1935) and the glutaric dialdehyde synthesis of ψ-pelletierine (Menzies & Robinson, 1924; Schöpf & Lehmann, 1935) and lobelanine (Schöpf & Lehmann, 1935).

[$^{14}C_2$]Ornithine has been found (Leete, Marion & Spenser, 1954; Haga, 1956) to act as a precursor of hyoscyamine, a tropane alkaloid, and the bridgehead carbon atoms have been found equally labelled (Haga, 1956). A compound of Δ′-pyrroline derived from ornithine can be postulated as an intermediate in the biosynthesis of tropane alkaloids such as hyoscyamine, equal labelling of the bridgehead carbon atoms of the tropane skeleton arising by double-bond migration in the Δ′-pyrroline compound. The fact that ornithine, but not 1:4-diaminobutane, serves as a precursor of hyoscyamine in *Datura stramonium* (Leete *et al.* 1954) could be due to the presence of an enzyme system forming the Δ′-pyrroline compound from ornithine but not from 1:4-diaminobutane.

The biosynthesis of nicotine can be postulated as taking place by the condensation of a vinylogue of a β-oxo acid, 5-carboxy-5-hydro-2-pyridone and Δ′-pyrroline. Such a route meets the requirements suggested by feeding experiments with labelled compounds (Dawson, Christman, D'Adamo, Solt & Wolf, 1958; Grimshaw & Marion, 1958). As with hyoscyamine the α- and α′-carbon atoms of the pyrrolidine ring of nicotine have been found equally labelled (Leete, 1955; Dewey, Byerrum & Ball, 1955) and tautomerism of the postulated intermediate Δ′-pyrroline could be the cause rather than symmetrical intermediates such as pyrrolidine (Dewey *et al.* 1955) or 1:4-diaminobutane (Leete, 1955). Anabasine might be formed

in a similar manner by condensation of 5-carboxy-5-hydro-2-pyridone with 2:3:4:5-tetrahydropyridine or 2:3:4:5-tetrahydropicolinic acid. [$^{14}C_2$]-Lysine was found, in feeding experiments with *Nicotiana glauca* (Leete, 1956), to act as a precursor of the piperidine ring of anabasine, although experiments with excized leaves showed only very slight incorporation of [$^{14}C_2$]lysine into anabasine (Aronoff, 1956). In the experiments of Leete (1956) all the ^{14}C activity of the anabasine was found at the single carbon atom of the piperidine ring which is attached to the pyridine. This was interpreted as being due to an asymmetrical precursor, possibly 3:4:5:6-tetrahydropicolinic acid, in a preceding reaction. More recently, Leete (1958) has shown that 1:5-diamino[1:5-$^{14}C_2$]pentane is a more efficient precursor (of the piperidine ring of anabasine) than [$^{14}C_2$]lysine. The 1:5-diaminopentane was considered to be converted into 2:3:4:5-tetrahydropyridine before incorporation into the anabasine molecule, which appears to be equally labelled at the $C_{(2)}$ and $C_{(6)}$ atoms of the piperidine ring. Nicotine, anabasine and *iso*pelletierine have been found to occur together in *Duboisia myoporoides* (Mortimer & Wilkinson, 1957), which suggests the possibility of similar biosynthetic routes.

The unsaturated ring compounds may also condense with nucleophilic species other than those derived from β-oxo acids. Thus van Tamelen & Knapp (1955) prepared β-(2-piperidyl)indole by condensing 2:3:4:5-tetrahydropyridine with indole and, in work on the synthesis of oxygenated tetracyclic lupin alkaloids, van Tamelen & Baran (1956) obtained 3-α-pyridylquinolizidine-1-carboxylic acid as the product of the reaction between 2:3:4:5-tetrahydropyridine and 2-(α-pyridyl)allylmalonic acid. Preliminary experiments suggest β-(2-piperidyl)indole is formed when the oxidation of 1:5-diaminopentane, catalysed by plant amine oxidase, is carried out in the presence of indole.

If alkaloids are waste products of metabolism it seems likely that they might be formed by non-specific mechanisms such as that described in the present work. The lag period in the production of carbon dioxide, which occurs with the amine oxidase reaction mixtures containing β-oxo acids, suggests that the condensation reactions are unlikely to occur to any great extent where rapid specific pathways exist for the metabolism of the unsaturated ring compounds. Recent work suggests that specific enzymes may exist which catalyse the direct addition of hydrogen to unsaturated compounds of this type to give pyrrolidine and piperidine compounds, and oxidation to aromatic ring compounds is a possible alternative metabolic pathway. Thus Δ′-pyrroline-5-carboxylic acid is reduced to proline by extracts of *Neurospora* (Yura & Vogel, 1955) and by rat-liver preparations (Smith & Greenberg, 1956, 1957). Meister & Buckley (1957) have shown that 3:4:5:6-tetrahydropicolinic acid is reduced to pipecolinic acid by enzyme preparations from rat or rabbit liver. The same preparations also catalyse the reduction of Δ′-pyrroline-2-carboxylic acid and Δ′-pyrroline-5-carboxylic acid to proline. The results of further investigations (Meister, Radhakrishnan & Buckley, 1957) suggest that different enzymes catalyse the reduction of each of the two last-named compounds and show that extracts of *Pisum sativum* and *Phaseolus radiatus* reduce 3:4:5:6-tetrahydropicolinic acid and Δ′-pyrroline-2-carboxylic acid to pipecolinic acid and proline respectively. Preparations of D-amino acid oxidase catalyse the formation of pyrrole-2-carboxylic acid from hydroxy-D- and *allo*hydroxy-D-proline (Radhakrishnan & Meister, 1956, 1957; Letellier & Bouthillier, 1956). Radhakrishnan & Meister (1957) also observed enzymic conversion of hydroxy-L-proline into pyrrole-2-carboxylic acid by intact- and broken-cell suspensions of a soil bacterium and obtained evidence that Δ′-pyrroline-4-hydroxy-2-carboxylic acid, which spontaneously forms pyrrole-2-carboxylic acid, is an intermediate in all three reactions. In a preliminary note Hasse & Berg (1957) report the oxidation of 1:5-diaminopentane to anabasine by pea-seedling extracts. The tetrahydroanabasine formed as a result of the action of the amine oxidase present is oxidized by a system of two components in the extracts, one of which can be replaced by Mn^{2+} ions. These considerations suggest the possibility that oxidations catalysed by amine oxidase may be stages in the biosynthesis not only of pyrrolidine and piperidine but also of pyrrole and pyridine compounds.

Lastly it should be pointed out that Krueger (1955) observed decarboxylation of β-oxo acids by *o*-quinones formed by the action of tyrosinase. The postulated mechanism of reaction involved condensation of the β-oxo acid with the *o*-quinone.

SUMMARY

1. The oxidation of 1:4-diaminobutane, 1:5-diaminopentane, 1:6-diaminohexane and 1:10-diaminodecane, catalysed by purified plant amine oxidase preparations in the presence of catalase, took place without formation of carbon dioxide. When these oxidations were carried out in the presence of β-oxo acids carbon dioxide was formed. The outputs of carbon dioxide sometimes exceeded the uptakes of oxygen and arose by decarboxylation of the β-oxo acids.

2. The rates of decarboxylation in such systems depended on the amine used as substrate. It was greatest with 1:4-diaminobutane and least with 1:10-diaminodecane.

3. Benzoylacetic acid, oxaloacetic acid and acetonedicarboxylic acid were decarboxylated more readily than was acetoacetic acid. The α-oxo acids tested were not decarboxylated.

4. In manometric experiments evidence was obtained that the decarboxylations were due to stoicheiometric reactions between the β-oxo acids and the oxidation products of the amines.

5. The results of paper-chromatographic investigations suggested that the decarboxylations were dependent on condensation reactions between the unsaturated ring compounds, formed by the spontaneous cyclization of the products of oxidation of the amines, and the β-oxo acids, which resulted in the formation of saturated ring compounds. This was established by the isolation of norhygrine and *iso*pelletierine from large-scale reaction mixtures in which 1:4-diaminobutane and 1:5-diaminopentane respectively were oxidized in the presence of acetoacetate.

6. The possible significance of the results in relation to alkaloid biosynthesis is discussed.

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Calcium and Magnesium Metabolism in Calves

4. BONE COMPOSITION IN MAGNESIUM DEFICIENCY AND THE CONTROL OF PLASMA MAGNESIUM*

By R. H. SMITH
National Institute for Research in Dairying, University of Reading, Shinfield, Berks

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Orent, Kruse & McCollum (1934) first showed that bone magnesium is depleted in rats kept on magnesium-deficient diets. This was subsequently also shown to be true for calves (Knoop, Krauss & Hayden, 1939; Blaxter, Rook & MacDonald, 1954; Blaxter & Sharman, 1955; Parr, 1957). It has also been shown that in neither of these animals are the soft tissues usually appreciably depleted even under the most severe conditions of magnesium deficiency (Cunningham, 1936; Blaxter *et al.* 1954; Parr, 1957; and our own unpublished observations) although MacIntyre & Davidsson (1958) have recently reported a small fall in skeletal-muscle magnesium in magnesium-deficient rats.

It appears therefore that bone magnesium represents a store of this element which can be called upon under conditions of deficiency to supply the needs of the soft tissues. It has been shown by killing rats at different times after introducing a magnesium-deficient diet that their bone magnesium can rapidly be mobilized (Duckworth, Godden & Warnock, 1940; Duckworth & Godden, 1941). Similar experiments with calves have not been carried out but Blaxter (1956) has related bone magnesium to plasma magnesium in calves slaughtered with differing degrees of hypomagnesaemia developed as a result of protracted milk feeding.

Our experiments on the development of hypomagnesaemia in milk-fed calves (Smith, 1957, 1958, 1959) made it desirable to follow associated changes in bone composition in such animals and other magnesium-deficient calves. The use of vertebrae taken from the tail of the living animal for this purpose will be described.

* Part 3: Smith (1959).

EXPERIMENTAL

Sample preparation and analysis

Bones examined were the caudal vertebrae, rib shaft, femur head and shaft and the first phalanges. They were removed either after slaughter or, with some of the caudal vertebrae, from sections of the tail taken from the living animal, a local anaesthetic (Nupercaine) being used. In this way at least ten successive samples could be obtained from one calf if sections containing only one vertebra were removed at a time. The bones were thoroughly cleaned of adhering soft tissue, cartilage and fat. Samples of the vertebrae and phalanges consisted of whole bones which were split longitudinally. Samples taken from the femur and rib shafts (which were cut transversely from about the middle of the bone) and from the femur head each weighed about 1–2 g. The samples were extracted in a Soxhlet extractor first for 12 hr. with ethanol and then for 6 hr. with ether. They were dried in an oven at 100° to constant weight and then ashed at 600° for about 18 hr. The ash was dissolved in 2N-HCl, the solution made up to a suitable volume and a 5 ml. sample containing about 0·05 mg. of magnesium added to a 15 ml. conical centrifuge tube. With methyl orange as an indicator saturated sodium acetate was added dropwise to give a pH of about 4–5. Calcium was then precipitated as the oxalate and titrated with permanganate; magnesium was precipitated as magnesium ammonium phosphate and determined colorimetrically. The method was that of Green & Allcroft as described by Godden (1937). The accurate determination, by chemical means, of magnesium in the presence of a large excess of calcium is difficult. We found that with the above method

for a solution containing magnesium and calcium in the ratio of 1:55 (about the ratio in normal bone) magnesium was recovered to an extent of 98·4 S.D. ±0·2% (six determinations). For a solution in which this ratio was 1:140 (about that in bone grossly depleted of magnesium) the magnesium recovery was 94·5 S.D. ±0·2% (six determinations). In view of the simplicity and good reproducibility of the method and since we were principally concerned with comparative measurements it was considered that this error could be tolerated. It should be remembered, however, that our figures for the percentage of magnesium in bone ash are probably too low by about 0·01–0·02.

Plasma magnesium and calcium were determined as described previously (Smith, 1957).

Treatment of animals

The bone composition in several calves fed normally and in two groups of calves developing hypomagnesaemia was examined. The first group developing hypomagnesaemia consisted of Shorthorn calves (nos. 1, 3, 4, 6, 7) which were fed almost exclusively on cow's milk supplemented when they got older with vitamin D, iron, copper and manganese. Some were also given magnesium supplements for part of the time. Caudal vertebrae were removed at approximately monthly intervals. These animals were also used for balance experiments etc., and further details of their treatment have been described previously (Smith, 1957). The second group consisted of four Friesian bull calves which, at about 2–4 weeks of age, were transferred from a magnesium-adequate diet (magnesium-supplemented synthetic milk or cow's milk, 4·40 l./day) to one of low-magnesium synthetic milk (4·40 l./day). Caudal vertebrae were removed before this change when the magnesium status of the calves was normal, and at intervals afterwards. The composition and method of preparation of the synthetic milk has been described previously (Smith, 1959). It contained about 0·6–0·8 mg. of magnesium/100 ml.

RESULTS

Caudal vertebrae as an index of bone composition

In order to examine the variability between different caudal vertebrae from any one calf the whole tails from a number of slaughtered calves showing different degrees of hypomagnesaemia were obtained. The individual vertebrae were analysed separately. The results given in Table 1 show that the composition of most of these vertebrae in an individual animal was closely similar and that any one could be used to assess, with reasonable accuracy, the composition of the remainder. This appeared to be true whatever degree of magnesium depletion had occurred. Indeed the variability seemed to be somewhat less in the hypomagnesaemic animals than in the normal animals but this was probably due to the fact that the former were older (about 8 months) than the latter (about 1 week). The results given in Table 1 do not include those for the very small bones at the tip of the tail. These tended to vary slightly in composition from the other caudal vertebrae (maximum observed variation in ash content about 12%). We avoided them in subsequent experiments by using only vertebrae with a dry weight greater than 0·1 g.

Table 2 shows the relation between the composition of the caudal vertebrae and the composition of certain other bones in normal and magnesium-depleted calves. Although the ash contents of the different bones were somewhat variable the only marked consistent difference was for the femur

Table 1. *Variability in the composition of different caudal vertebrae from individual calves*

Each column gives the results for an individual calf. Mean values for each calf are given with the standard deviations for determinations on individual vertebrae in parentheses.

	Normal		Magnesium-depleted		
No. of vertebrae	9	11	12	12	11
Ash in dry bone (%)	—	52·2 (1·6)	57·1 (0·9)	59·3 (0·5)	59·4 (0·7)
Magnesium in ash (%)	0·751 (0·026)	0·825 (0·022)	0·401 (0·006)	0·386 (0·009)	0·236 (0·009)
Calcium in ash (%)	38·5 (0·4)	37·9 (0·5)	39·4 (0·3)	39·2 (0·3)	39·2 (0·2)

Table 2. *Relationship between the composition of the caudal vertebrae and the composition of certain other bones in normal and magnesium-depleted calves*

Composition of each of the different bones is expressed as a percentage of the composition of the caudal vertebrae from the same animal. Mean values (±S.D.) are given for two normal calves (mean caudal-vertebrae ash in dry bone 53·3%, calcium in ash 38·6% and magnesium in ash 0·73%) and for three magnesium-depleted calves (mean caudal-vertebrae ash in dry bone 58·5%, calcium in ash 39·1% and magnesium in ash 0·37%).

		Phalanges	Femur head	Rib shaft	Femur shaft
Ash in dry bone	Normal	106±3	98±1	111±8	130±7
	Depleted	102±2	104±3	99±5	123±2
Calcium in ash	Normal	101±0	99±1	101±1	101±0
	Depleted	100±2	100±1	100±1	99±1
Magnesium in ash	Normal	99±1	101±3	101±3	101±5
	Depleted	101±5	109±2	115±7	137±13

Table 3. *Composition of the caudal vertebrae in calves of different ages and with different degrees of magnesium depletion*

Mean results are given (±S.D.).

	Normal		Magnesium moderately depleted	Magnesium grossly depleted
No. of calves	5	3	3	3
Age (weeks)	1–3	30–40	30–40	30–40
Ash in dry bone (%)	55·4±1·1	58·3±1·0	58·6±0·7	58·5±0·6
Calcium in ash (%)	38·9±0·5	39·1±0·3	39·0±0·3	39·3±0·2
Magnesium in ash (%)	0·76±0·02	0·71±0·03	0·43±0·04	0·29±0·04

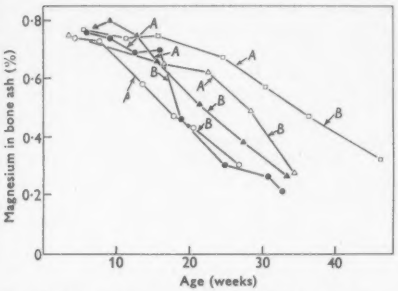

Fig. 1. Relation between bone (caudal vertebrae) magnesium and age in calves fed on cow's milk and developing hypomagnesaemia at different rates. ○, Calf 1; ●, calf 3; □, calf 4; △, calf 6; ▲, calf 7. Ages at which the different calves showed concentrations of plasma magnesium of 1·6 and 0·7 mg./100 ml. are indicated by arrows labelled *A* and *B* respectively.

shaft. This contained about 20–30 % more ash than did the caudal vertebrae in both the normal and magnesium-depleted calves. The calcium in the ash was closely similar for the different bones in all the calves, as was the magnesium in the ash for the normal calves. In the magnesium-depleted calves, however, there were marked differences in the magnesium contents of the ashes of the different bones. Thus although magnesium depletion in these calves occurred in all the bones examined it did not occur to the same extent in all of them. Of those studied the femur shafts were depleted to the least extent and the vertebrae and first phalanges to the greatest extent. It appears therefore that the caudal vertebrae offer a sensitive guide to the magnesium status of the skeleton but that changes observed in these vertebrae during magnesium depletion do not exactly reflect the changes occurring in the skeleton as a whole.

Bone composition in magnesium deficiency

The results given in Table 3 show some small differences in composition between caudal vertebrae from calves of different ages. These differences were not, however, statistically significant and the difference for magnesium in the ash was in any case so small as to be of little importance in relation to changes occurring in magnesium deficiency. The changes in bone-ash magnesium for calves receiving cow's milk, shown in Fig. 1, can be regarded therefore as reflecting changes in the magnesium status of the animals and were not to any appreciable extent due intrinsically to the increasing age of the calves.

There appeared usually to be a broad direct relationship between the rate of onset of hypomagnesaemia in the different calves (itself probably controlled by the rate of fall of efficiency of magnesium absorption, Smith, 1957, 1958, 1959) and the rate of depletion of their bone magnesium. Thus with one exception (calf no. 3, which will be discussed below) falls to concentrations of plasma magnesium of 1·6 and 0·7 mg./100 ml. were associated with bone-ash magnesium figures of 0·60–0·67 and 0·40–0·48 % respectively, whatever the rate at which the concentration of plasma magnesium fell. The plasma magnesium decreased only slowly after it reached about 0·7 mg./100 ml. (see for example Fig. 2 of Smith, 1957) and further depletion of bone magnesium occurred with only a small decrease in this concentration. When the last bone samples were taken, at or within 2 weeks of death (the calves either died, presumably from magnesium deficiency, or were slaughtered with severe symptoms of magnesium deficiency), the mean plasma magnesium level was 0·47 S.D. ± 0·05 mg./100 ml., and the mean magnesium in the bone ash was 0·27 S.D. ± 0·02 % (about 38 % normal). The relationship between plasma magnesium and bone magnesium in these calves is illustrated in Fig. 2.

Fig. 3 shows the changes occurring in bone magnesium and plasma magnesium after the abrupt onset of an acute condition of magnesium deficiency brought about in a group of calves by replacing a magnesium-adequate diet with one of low-magnesium synthetic milk. These results are expressed as percentages of the starting (normal) values since the different calves varied somewhat in their normal levels. After the introduction of the

magnesium-deficient diet, the depletion of bone magnesium appeared to occur at an approximately constant rate, presumably at a rate necessary to provide adequate magnesium for the growing soft tissue and for endogenous faecal excretion [urine excretion falls nearly to zero as soon as calves are transferred to a magnesium-deficient diet (Smith, 1959)]. Plasma magnesium fell, rapidly at first, and then more slowly. The growth rate of the calves, and their consequent need for magnesium, was depressed when they received synthetic milk (average weight increase about 1 kg./week compared with about 2 kg./week on a similar amount of cow's milk) and the effects of the magnesium deficiency were consequently less dramatic than might have been expected. The relation between plasma magnesium and bone magnesium for these calves is also illustrated in Fig. 2.

When the bone-ash magnesium values of two of the calves fed on low-magnesium synthetic milk had fallen to 84 and 68% respectively of the normal value they were given daily subcutaneous injections of 1 g. of magnesium (as the sulphate). After 5 days of injections the plasma magnesium of both calves ranged between about 3·7 and 2·2 mg./100 ml. (about 160–95% of normal) after each injection. After 9 days of injections in one case and 16 days of injections in the other the bone-ash magnesium for both calves was found to be normal (each was 102% of the value obtained before the magnesium-deficient diet was fed).

The bone samples taken periodically from each of the calves used in the above experiments did not show any marked changes in ash content or in the percentage of calcium in the ash concomitant with magnesium depletion. Some of the calves showed evidence of faulty calcium utilization for part of the time, due apparently to vitamin D deficiency (Smith, 1957), but bone samples were not usually taken during these periods. Calf 7 showed some indication of slightly reduced bone ash (reduced by about 5–10%) in two samples taken when the calf was hypocalcaemic, but no other apparent abnormalities were observed. The results in Table 3 all refer to calves receiving adequate vitamin D. They show no statistically significant differences in bone-ash content or in the percentage of calcium in the ash between calves of similar age but widely differing magnesium status.

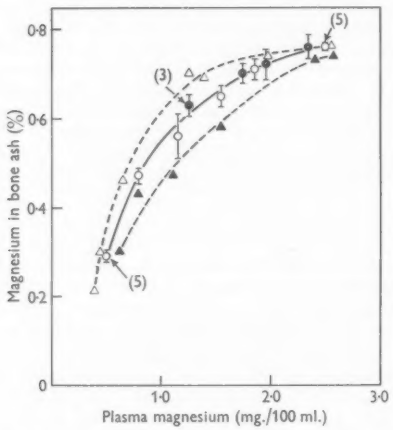

Fig. 2. Relation between bone (caudal vertebrae) magnesium and plasma magnesium in calves developing hypomagnesaemia. Mean results (●, ○) were for groups of calves in each of which the plasma-magnesium values did not differ by more than ±0·15 mg./100 ml. from the mean. Standard deviations for the corresponding bone-ash magnesium values are shown as vertical bars. There were four animals in each group except for those in which the number of animals has been shown by a figure in parentheses. Calves fed on cow's milk and low-magnesium synthetic milk are indicated by ○ and ● respectively. Results for two individual calves are also given and are indicated by the symbols ▲ (calf no. 1) and △ (calf no. 3) with the appropriate curves shown as broken lines.

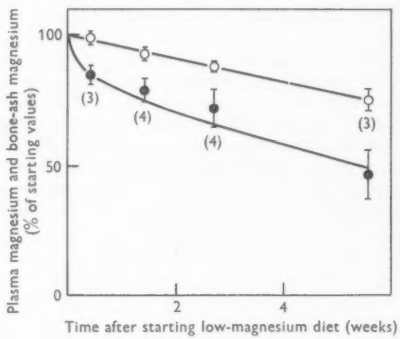

Fig. 3. Changes in bone (caudal vertebrae)-ash magnesium (○) and plasma magnesium (●) after an abrupt change from a magnesium-adequate diet to one of low-magnesium synthetic milk. The calves were 2–4 weeks old at the time of the change in diet. The results given are mean values (with standard deviations shown as vertical bars and the numbers of calves in parentheses) expressed as a percentage of the initial values obtaining on the magnesium-adequate diet. These initial values (mean results for four calves) were 2·34 S.D. ±0·06 mg./100 ml. for plasma magnesium and 0·76 S.D. ±0·03% for bone-ash magnesium.

DISCUSSION

The removal of caudal vertebrae appears to provide a satisfactory means of assessing bone composition in the living calf showing magnesium deficiency (and could probably also be used in studying bone composition in other conditions). The method offers obvious advantages over rib biopsy, which has been used by Thomas & Okamoto (1958) to assess bone magnesium. It should be borne in mind, however, that although in the normal animal we found magnesium to be uniformly distributed in the ash of the different bones which we examined, in magnesium deficiency this distribution was not uniform. This finding is not in agreement with the conclusions of Blaxter (1956). However, these conclusions were based on comparisons between bones (rib shaft, rib end and metatarsal shaft) different from those which we examined. Moreover, it is possible that in the final stages of depletion the bones which lose magnesium more rapidly reach a minimum value, while the bones losing magnesium more slowly catch up. Thus although in moderate depletion such as we examined differences exist, in gross depletion they may tend to disappear.

Our results suggest that magnesium behaves similarly to calcium in the lack of uniformity in depletion of different parts of the skeleton during deficiency. Thus calcium has been shown to be removed more readily from cancellous bone (e.g. vertebrae) than from compact bone (e.g. femur shaft) in calcium-deficient sheep (Benzie, Boyne, Dalgarno, Duckworth, Hill & Walker, 1955).

The relationship between bone and plasma magnesium shown in Fig. 2 is similar in general form to that reported by Blaxter (1956) for data obtained from calves slaughtered with varying degrees of hypomagnesaemia developed as a result of milk feeding. Such absolute differences as exist may be partially accounted for by the fact that Blaxter (1956) examined bone from the metatarsal shaft whereas we used caudal vertebrae.

The relationship suggests that in hypomagnesaemia an equilibrium exists between bone and plasma magnesium. It also suggests that normal bone, from a physiological standpoint, is virtually saturated with magnesium. This was further indicated by the fact that daily injection of magnesium into two calves, so that the plasma magnesium did not fall appreciably below normal and for part of the time was considerably above normal, did not lead to any appreciable increase in bone magnesium above normal amounts. Thus it appears that under normal conditions, when excess of magnesium is entering the blood, the bone plays little or no part in regulating concentration of plasma magnesium. This control appears to be largely effected by changes in urine excretion. This excretion is high when the diet contains more than adequate magnesium (Smith, 1957, 1958), increases enormously when magnesium is injected so that plasma magnesium rises above normal (Smith, 1959), and decreases, eventually nearly to zero, when the magnesium available from the diet decreases (Smith, 1957, 1958). When the available magnesium is so low as to be inadequate even with almost no loss in the urine then bone magnesium is apparently liberated. Under these conditions plasma magnesium is not maintained at a normal concentration, the liberated magnesium is utilized elsewhere and as the bone becomes more and more depleted plasma magnesium drops to maintain the relationship shown in Fig. 2. After depletion has started injection of a small amount of magnesium causes an increase in plasma magnesium, which is followed by a drop nearly to the previous depleted amount (unpublished results). Urine excretion does not increase appreciably if the plasma magnesium remains below normal and the drop is presumably due to magnesium being taken up by the bone to restore the equilibrium. Our present work shows that repeated injection eventually restores both bone magnesium and plasma magnesium to normal.

All the animals studied showed a relationship between bone and plasma magnesium similar in general form to the mean curve shown in Fig. 2. Different animals, however, showed marked differences of detail; extreme differences were shown by calves nos. 1 and 3 respectively and the individual curves for these calves are also shown in Fig. 2. Such differences must affect the rate of fall of plasma magnesium during a period of magnesium deficiency. For example, the depletion of say 13% of the bone magnesium in calves nos. 1 and 3 would lead to the animals showing plasma-magnesium levels of about 1·8 and 1·1 mg./100 ml. respectively. Plasma magnesium appears therefore to be only an approximate guide to the magnesium status of any individual animal. This helps to explain apparent anomalies in the different rates at which different milk-fed calves develop hypomagnesaemia, for although such different rates can usually be broadly related to corresponding differences in the efficiency with which the calves utilize dietary magnesium (Smith, 1957, 1958) this does not represent the whole picture. Thus, for example, calves nos. 1 and 3 on a milk diet showed almost identical rates of fall of plasma magnesium (that for calf no. 1 is given in Fig. 2 of Smith, 1957) and yet calf no. 1 showed a considerably more rapid decrease in utilization of dietary magnesium (Smith, 1957). These facts can be reconciled by the above considerations.

Shortly before death or the appearance of serious clinical symptoms of magnesium deficiency our calves all showed values of about 0·2–0·3% of

magnesium in the bone ash (about 60–70% depletion). Similar values at death from magnesium deficiency have also been reported by Blaxter & Sharman (1955). These values appear to correspond approximately with the first achievement of a lower limiting level for plasma magnesium of about 0·3–0·5 mg./100 ml. (Fig. 2). Dallemagne & Fabry (1956) have suggested that about two-thirds of bone magnesium is adsorbed on the surface of the bone-salt crystals, with the remaining one-third replacing calcium in the phosphate molecule. It seems possible that the adsorbed fraction is, in fact, that part which is available in magnesium depletion. If this were so the plasma magnesium, about 0·3–0·5 mg./100 ml., would correspond to the virtually complete removal of this adsorbed magnesium. This concentration may therefore be that at which there is magnesium equilibrium between some other tissue or tissues and the extracellular fluid.

Although our results showed no marked change in bone calcium associated with magnesium depletion they were too few to give any reliable information on the small change which would result from the replacement of magnesium by calcium in the bone salt as suggested by Blaxter (1956).

SUMMARY

1. Individual caudal vertebrae have been shown to provide a satisfactory guide to the composition of the skeleton in magnesium-deficient calves. The depletion of bone magnesium occurring in this condition was uniform in the different caudal vertebrae although it varied somewhat in other parts of the skeleton.

2. Periodic removal and examination of these vertebrae from the living calf have been used to follow changes in the magnesium status of the skeleton during conditions of magnesium deficiency resulting from feeding with cow's milk or low-magnesium synthetic milk. Bone-ash magnesium fell progressively from a normal figure of about 0·75% eventually to about one-third of this value when serious clinical symptoms of magnesium deficiency became manifest. Injection of sufficient magnesium readily restored normal values.

3. A relationship between bone-ash magnesium and plasma magnesium has been shown. The evidence suggests that the bone plays little or no part in the control of plasma magnesium under conditions of magnesium excess but that in magnesium deficiency in calves the concentration of plasma magnesium is determined by the concentration of bone magnesium.

4. No significant changes in total bone ash or in the proportion of calcium in the ash were observed concomitant with the depletion of bone magnesium.

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Lecithinase and Lysolecithinase of Intestinal Mucosa

By B. EPSTEIN* and B. SHAPIRO
Department of Biochemistry, Hebrew University-Hadassah Medical School, Jerusalem, Israel

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Enzymes, capable of hydrolysing lecithin to liberate inorganic phosphate, are present in the kidney and intestinal mucosa (King, 1931). Since several pathways may lead from lecithin to inorganic phosphate and more than a single enzyme must have been involved, it is uncertain which enzyme was the rate-limiting one in this investigation. In a recent paper Schmidt, Bessman & Thannhauser (1957) described a mitochondrial preparation of rat intestinal mucosa, which catalysed the splitting of fatty acids from kephalin. Some activity, though a much smaller one, was found with lecithin as substrate. The low activity of similar intestinal-mucosa preparations towards lecithin was attributed by us, in a preliminary communication (Epstein & Shapiro, 1957), to the fact that lecithin degradation by the intestinal enzyme depends upon the presence of fatty acids. This activation by one of its reaction products puts the enzyme involved into a category apart from other lecithinases. As will be seen in the present paper, intestinal lecithinase differs from known lecithinases in several other respects.

EXPERIMENTAL

Materials

Ovolecithin. This was prepared according to Hanahan, Turner & Jayko (1951). A 0·5% (w/v) suspension was prepared by mixing the lecithin with hot water in a Waring Blendor. The suspension was kept frozen.

Lysolecithin. After preparation according to Belfanti (1941), 10 mg. was suspended in 1 ml. of water. The suspension was kept frozen.

Acyl ester. Content of acyl ester was determined according to Shapiro (1953).

L-α-*Glycerylphosphorylcholine.* The cadmium salt of this substance was kindly supplied by Dr G. Schmidt and was freed of cadmium according to Tattrie & McArthur (1955). It was determined in reaction mixtures according to Shapiro (1953).

Fatty acids. The acids were dispersed in boiling water to give a 25 mM-solution of pH 8–9 after addition of 0·1N-KOH. The solution was kept in a refrigerator and was warmed before use to dissolve the fatty acids. Their concentration in the reaction mixtures was determined by titration according to Dole (1956).

Albumin. Bovine serum albumin (Pentex Co. Incorp., Kankakee, Ill., U.S.A.) was used.

Protein. This was determined by a modification of the method of Herriot (1941) according to Kaufman & Wertheimer (1957).

Enzyme preparations

Preparation of S_1 and P_1. Rats were killed by decapitation, the small intestines were removed and rinsed through with 0·9% NaCl soln. After the intestines were cut open, the mucosa was scraped off with a blunt spatula. In all the following steps the preparation was kept below 5°. The mucosa was ground with an equal weight of sand, and with water (2 ml./g. of mucosa); the mixture was then centrifuged at 800 *g* for 5 min. The supernatant (S_1) was brought to pH 5·6 with 0·1N-acetic acid or 0·1N-HCl, centrifuged at 12 000 *g* and the precipitate (P_1) was resuspended in 0·02M-sodium phosphate buffer (2 ml./g. of original mucosa) so that the final pH was 6·5.

'Mitochondrial' and 'microsomal' fractions. The mucosa was homogenized in a tight-fitting glass homogenizer with 10 vol. of 1·15% (w/v) KCl. In all the following steps the preparation was kept below 5°. The suspension was centrifuged at 12 000 *g* for 20 min. The precipitate was homogenized again with 5 vol. of 1·15% KCl and centrifuged at 700 *g* for 5 min. The supernatant was centrifuged at 12 000 *g* for 20 min. and the precipitate, called the 'mitochondrial fraction', was resuspended in 0·02M-sodium-phosphate buffer (1 ml./g. of original mucosa), so that the final pH was 6·5. The first and last supernatant fractions were combined and re-centrifuged at 40 000 *g* in a Spinco centrifuge; the resulting precipitate, called the 'microsomal fraction', was resuspended as above.

Reaction mixtures. Unless otherwise stated these were composed of 5 μmoles of lecithin, 5 μmoles of sodium oleate, 40 μmoles of sodium phosphate buffer, pH 6·5, and 0·3 ml. of P_1 and were made up to 1 ml.

With lysolecithin, 7 μmoles of substrate and 0·1 ml. of P_1 were used.

Incubation. This was at 37° for the indicated time intervals and the reaction was stopped by adding 4 ml. of trichloroacetic acid soln. (5%, w/v) for glycerylphosphorylcholine estimation or with 4 ml. of ethanol–ether mixture (3:1, v/v) for determination of the acyl ester content.

RESULTS

As already reported in our preliminary note (Epstein & Shapiro, 1957) breakdown of lecithin with the enzyme preparation described was preceded by a lag phase. This lag phase could be abolished by increasing the enzyme concentration or by adding fatty acids (Fig. 1).

* Part of Ph.D. thesis submitted to the Senate of the Hebrew University, Jerusalem, Israel.

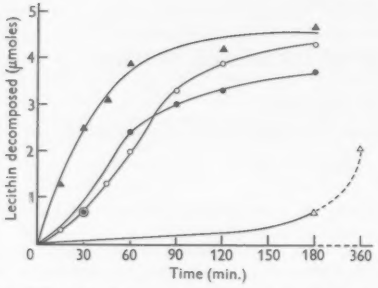

Fig. 1. Effect of palmitic acid and enzyme concentration on intestinal lecithinase activity. △, With 0·3 ml. of enzyme; ○, with 0·3 ml. of enzyme + 4 μmoles of palmitic acid; ●, with 0·6 ml. of enzyme; ▲, with 0·6 ml. of enzyme + 4 μmoles of palmitic acid. The rate of reaction was determined by following the decrease in acyl ester content.

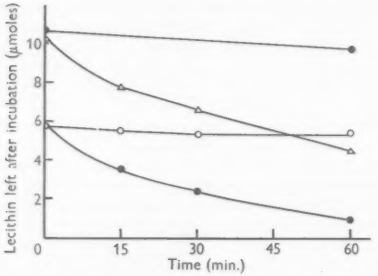

Fig. 2. Effect of lecithin concentration on the concentration of oleic acid required for activation. ○, Without added oleic acid; ●, with 5 μmoles of oleic acid; △, with 10 μmoles of oleic acid.

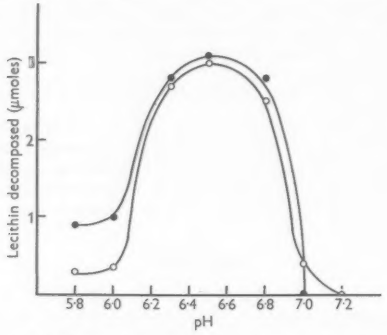

Fig. 3. pH–Enzyme activity curve of intestinal lecithinase in the presence of oleic acid. ○, Glycerylphosphorylcholine formation; ●, decrease in ester content. Time of reaction: 45 min.

Table 1. *Effectiveness of various fatty acids in shortening the lag period of lecithin degradation*

Fatty acid (5 μmoles) added	Time required for the splitting of 1·4 μmole of lecithin (min.)
Linoleic acid	10
Oleic acid	10
Lauric acid	26
Myristic acid	58
Decanoic acid	96
Palmitic acid	144
Stearic acid	177
Octanoic acid	196
Hexanoic acid	210
Butyric acid	246
Acetic acid	300
Without acid	360

Table 2. *Breakdown of lecithin and lysolecithin by various enzyme preparations from intestinal mucosa*

Enzyme system	Hydrolysis of substrate (μmoles/hr./mg. of protein) With lecithin	With lysolecithin
S_1	0·84	7·0
P_1	1·84	10·0
'Microsomes'	1·60	20·0
'Mitochondria'	1·10	12·4

The amount of fatty acid required for optimum activity was tested and it was found that this point was reached when amounts nearly equimolar to those of the lecithin were added. When the lecithin concentration was increased to 10 μmoles/ml. the usual amount of oleate (5 μmoles/ml.) was not enough to start the reaction and larger amounts of fatty acid were required (Fig. 2).

The effectiveness of the various homologous fatty acids in shortening the lag period was tested (Table 1). Oleic and linoleic acid were found to be the most active and the only acids which abolished the lag completely (the shortest time tested was 10 min.). The order of effectiveness in shortening the lag period in the saturated fatty acids series tested was:

$$C_{12} > C_{14} > C_{10} > C_{16} > C_{18} > C_8 > C_6 > C_4 > C_2.$$

In all subsequent experiments reported in this paper oleic acid was used.

The pH–enzyme activity curve in Fig. 3 shows a very sharp pH optimum at 6·5 and little activity at pH 6 or pH 7.

Generally close agreement was found between the decrease in ester content and the formation of glycerylphosphorylcholine. This is due to the large excess of lysolecithin-splitting enzyme in all the preparations used (Table 2). Lysolecithin breakdown proceeded without addition of fatty acid and

the rate of the reaction was unaffected by increasing the substrate concentration from 5 to 10 mM.

Endogenous fatty acids as activators. The activity found with the intestinal lecithinase in the absence of external fatty acids and the shortening of the lag period by increasing the enzyme concentration (Fig. 1) were thought to be due to endogenous fatty acids in the enzyme preparation. Estimation of free fatty acids in the enzyme preparation revealed that it contained 7 μmoles/ml. (the high content of endogenous fatty acids is due to hydrolysis of endogenous lipids during the preparation and storage of the enzyme). Various methods were tried to remove the endogenous fatty acids from the enzyme. Extraction of fresh mucosa, S_1 or lyophilized S_1, with acetone was found to inactivate the enzyme irreversibly; lyophilization, itself, had no detrimental effect on the activity. On the other hand, the endogenous fatty acids could be bound and inactivated by the addition of albumin or rat serum to the reaction mixture (Table 3) and finally it was shown that by washing P_1 with albumin solution the endogenous fatty acid content could be decreased to 0·5 μmole/ml. (Table 4) and only negligible lecithin breakdown could be demonstrated even after incubation for 24 hr. Addition of oleic acid to the albumin-washed enzyme restored about 50 % of the original activity, but butyric acid had no such activating effect.

Effect of metals on intestinal lecithinase. This is shown in Table 5. Hg^{2+}, Cd^{2+} and Ca^{2+} ions had a considerable inhibitory effect at the concentrations used. Ca^{2+} ion was not required for activity and the hydrolysis was not impaired by the addition of sodium ethylenediaminetetra-acetate (EDTA), pH 6·5, or by dialysis for 24 hr. against 0·02 M-sodium phosphate buffer, pH 6·5, at 5°. The inhibition by 0·4 mM-Cd^{2+} ion was not reduced by doubling the amount of oleic acid or lecithin. On the other hand, it could be reversed with EDTA (Table 6). Even after removal of possible inhibitory cations in the reaction mixture, the enzyme activity was still dependent on fatty acids.

Stability of lecithinase and lysolecithinase. This was examined when the compounds were incubated at 30° at various pH values. It was found that the enzymes were the most stable at pH 6·5 and that at the other pH values the activity dropped (Table 7).

Heat stability of intestinal lecithinase and lysolecithinase at pH 6·5. This is shown in Table 8. Heating the enzyme to 50° for 10 min. caused 100 % inactivation, at 40° for 10 min. 50% inactivation. Lecithin protected the enzyme from heat-inactivation and pre-incubation at 40° for 10 min. with lecithin even caused some activation. Since pre-incubation with lecithin was carried out without added fatty acids, no detectable hydrolysis occurred during this period. Lysolecithinase activity followed the same stability pattern as intestinal lecithinase activity.

Table 3. *Influence of albumin and rat serum on lecithin breakdown by intestinal lecithinase*

Addition	Hydrolysis (μmoles of lecithin/45 min.)
None	3·4
Albumin soln. 7·5%	
0·1 ml.	3·1
0·2 ml.	0·6
0·3 ml.	0
Rat serum	
0·1 ml.	3·3
0·2 ml.	2·4
0·3 ml.	0

Table 4. *Removal of endogenous fatty acids by washing with albumin solution*

Expt. no. 1. Albumin solution (30 %, w/v) was brought to pH 5·6 with 0·1 N-acetic acid; 4 ml. of this solution was mixed with 4 ml. of P_1 in a glass homogenizer. The mixture was centrifuged at 13 000 *g*. Residual albumin was removed by treating the resulting precipitate as above with 4 ml. of 0·02 M-sodium phosphate buffer, pH 5·6. The final precipitate was resuspended in 4 ml. of 0·02 M-sodium phosphate buffer, pH 6·5. Expt. no. 2. As in Expt. no. 1 except that P_1 was washed with 16 ml. of albumin solution (8 %, w/v).

Expt. no.	Enzyme system	Fatty acid (μmoles/ml. of enzyme)	Hydrolysis: Without added oleate (μmoles of lecithin/ 24 hr.)	Hydrolysis: With 5 μmoles of oleate (μmoles of lecithin/ 60 min.)	Hydrolysis: With 5 μmoles of oleate (μmoles of lecithin/60 min./ mg. of protein)
1	P_1	7	—	3·6	—
	Washed P_1	0·5	0·4	1·5	—
2	P_1	7	2·1	2·7	0·93
	Washed P_1	0·7	0·5	1·3	0·68
			0·4*	—	—

* With 5 μmoles of butyric acid.

Effect of detergents. Oleic acid could not be replaced by detergents such as Tween 80, cholic acid or saponin. The first two substances were even found to be inhibitory in the presence of added oleic acid (Table 9).

DISCUSSION

The obligatory requirement of fatty acids for the activity of intestinal lecithinase presents an unusual case of activation of an enzyme by its reaction product. The mechanism of this activation is not yet clear. It is not due merely to an emulsifying action of the fatty acids, since much more efficient emulsifiers, such as Tween 80 or cholic acid, are devoid of activity. The fact that the amount of fatty acid required for optimum activity depends upon the amount of substrate, and is roughly equimolar to it, points to a substrate–activator complex as a possible basis of activation. An alternative explanation that fatty acids may bind inhibitory cations, such as Cd^{2+} or Ca^{2+} ions, is unlikely, since these ions retained their inhibitory effect in the presence of excess of fatty acids and, moreover, after removal of Ca^{2+} ion inhibition by treatment with EDTA, fatty acids were still required for activity.

The enzyme obtained from intestinal mucosa differed from other lecithinases in several other respects. It was bound to particles, was highly

Table 5. *Influence of various metals on intestinal lecithinase activity*

Incubation was carried out for 45 min. Breakdown of lecithin without addition of metal was 3·4 μmoles.

Cation added	Concn. (mM)	Inhibition (%)
Hg^{2+}	0·1	14
Hg^{2+}	0·4	100
Cd^{2+}	0·4	70
Cu^{2+}	0·4	30
Zn^{2+}	0·4	20
Ca^{2+}	5·0	50
Fe^{3+}	3·0	26
Ni^{2+}	4·0	9
Co^{2+}	4·0	9

Table 6. *Influence of sodium ethylenediaminetetraacetate on inhibition by cadmium ions*

Breakdown of lecithin without addition of metal: 2·7 μmoles/45 min.

Addition to reaction mixture (without oleate)	Relative activity
5 μmoles of oleic acid	100
5 μmoles of oleic acid + 0·4 μmole of Cd^{2+}	55
5 μmoles of oleic acid + 1 μmole of EDTA	100
5 μmoles of oleic acid + 0·4 μmole of Cd^{2+} + 1 μmole of EDTA	100
1 μmole of EDTA	0

Table 7. *Stability of the enzyme at 30° at various pH values*

Enzyme (0·3 ml.) was pre-incubated at the indicated pH values for 30 min. at 30° and the reaction mixture was then readjusted to pH 6·5. Hydrolysis of the substrates without pre-incubation was 3·5 μmoles/45 min. for lecithin and 4·0 μmoles/20 min. for lysolecithin. The relative activities are percentages of these values.

pH of pre-incubation	Relative activity: With lecithin	Relative activity: With lysolecithin
5·5	20	—
5·7	74	72
6·0	83	89
6·5	100	100
6·7	94	—
7·0	83	—
7·5	77	55
8·0	23	—

Table 8. *Stability of the enzyme to pre-incubation at various temperatures*

Enzyme (0·3 ml.), with or without lecithin, was pre-incubated at pH 6·5 as indicated; the reaction mixture was then completed. Incubation time and hydrolysis without pre-incubation was 2·6 μmoles/45 min. for lecithin and 5·0 μmoles/20 min. for lysolecithin. Results are expressed relative to the hydrolysis after pre-incubation for 30 min. at 30°.

Temperature	Time (min.)	Relative activity: With lecithin: Pre-incubated without substrate	Relative activity: With lecithin: Pre-incubated with substrate	Relative activity: With lysolecithin. Pre-incubated without substrate
30°	30	100	—	—
37	30	27	142	—
40	10	50	150	88
45	10	23	84	62
50	10	0	19	11
55	10	0	11	6

Table 9. *Effect of detergents on intestinal lecithinase*

Addition to reaction mixture (without oleate)	Hydrolysis (μmoles of lecithin/45 min.)
4 μmoles of oleic acid	2·6
4 μmoles of Tween 80	0
4 μmoles of Tween 80 + 4 μmoles of oleic acid	0
1 μmole of Tween 80	0
1 μmole of Tween 80 + 4 μmoles of oleic acid	2·6
1 mg. of saponin	0·2
5 μmoles of cholic acid + 4 μmoles of oleic acid	1·0

thermolabile and did not require Ca^{2+} ions as treatment with EDTA or dialysis had no effect on the activity.

Lysolecithinase activity was not separated from the lecithinase by the procedures used. Both enzymes showed similar pH-dependence and heat-susceptibility. However, lysolecithinase did not require fatty acids for activity. Contrary to the findings with pancreas lysolecithinase (Shapiro, 1953) and liver lysolecithinase (Dawson, 1956), no self-inhibition by the substrate was found with intestinal lysolecithinase up to a concentration of 10 mM-lysolecithin. It is of significance that lysolecithinase activity was always much greater than that of the lecithinase. No considerable amounts of lysolecithin can therefore accumulate during splitting of lecithin in the intestinal mucosa.

It may be of significance for the biological function of intestinal lecithinase that the optimum conditions for its activity are those which can be expected to prevail in the mucosal cells during absorption of fat, i.e. an abundance of free fatty acids. On the other hand, the conditions in the intestinal lumen, which favour lipase action, namely the presence of bile acids and Ca^{2+} ions, are unsuitable for lecithinase activity.

SUMMARY

1. A particulate enzyme system which decomposes lecithin and lysolecithin into glycerylphosphorylcholine and fatty acids was isolated from the mucosa of rat intestines.

2. Lecithin breakdown was found to depend upon the presence of fatty acids. The optimum concentration of fatty acids depends upon the concentration of the substrate and is roughly equimolar to it. With suboptimum concentrations (e.g. with the endogenous fatty acids present in the enzyme preparation) the reaction is autocatalytic, starting with a lag period. This lag period can be prolonged to more than 24 hr. by removing most of the endogenous fatty acids from the enzyme by washing it with serum albumin solution.

3. Linoleic and oleic acid were the most efficient activators found, followed by lauric and decanoic acid of the saturated acid series. Tween 80, cholic acid and saponin could not replace fatty acids.

4. Ca^{2+} ion was not required for activity and was inhibitory at a concentration of 5 mM. Other bivalent cations, especially Hg^{2+} and Cd^{2+} ions, were also strongly inhibitory.

5. Lecithinase and lysolecithinase action had a pH optimum at 6·5 which was also the point of greatest stability. The enzyme was inactivated by short incubations at 30–50° but lecithin gave some protection.

6. Activity with lysolecithin was always greater than with lecithin and no fatty acids were required for breakdown of the former compound.

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Properties and Specificity of Pancreatic Phospholipase A

By A. RIMON* and B. SHAPIRO
Department of Biochemistry, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

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The hydrolysis of lecithin by pancreatic juice and extracts has been the subject of many investigations. However, the results obtained by various authors are contradictory (see Ercoli, 1940): whereas some failed to find any activity, others found more or less active splitting of lecithin. The contradictions seem to be due partly to the various preparations used (fresh pancreas, partly autolysed tissue, pancreatic juice, pancreatin preparations, etc.) or to the inadequacy of analytical methods.

In more recent investigations, Schmidt, Hershman & Thannhauser (1945) isolated glycerylphosphorylcholine from autolysing ox pancreas, and Shapiro (1952) demonstrated the presence in acetone-dried powders of ox pancreas of two enzymes, which split lecithin and lysolecithin into glycerylphosphorylcholine. The lysolecithinase was purified to a high degree (Shapiro, 1953), but the mode of action of the lecithin-splitting enzyme remained unclear.

Hanahan (1952) reported that pancreatic phospholipase A could split lecithin in ether solution. This enzyme was found in extracts of fresh rat pancreas or in commercial pancreatin preparations. Working with fresh ox pancreas, we were unable to extract an active enzyme nor could any lecithinase activity be demonstrated in the homogenate of the fresh tissue. However, upon storage of the frozen pancreas for at least 2 weeks, phospholipase activity appeared. This activity was confined to the insoluble particles.

This paper deals with the purification of this enzyme, its properties and its specificity. The substrates tested were phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine, which are generally regarded as substrates of phospholipase A (Zeller, 1950). In addition, phosphatidic acid, which was reported in a preliminary note (Rimon, Garcia & Shapiro, 1957) to be split by the pancreatic enzyme, was also included in this study.

EXPERIMENTAL

Materials. Phosphatidyl choline (PC) was prepared from egg yolk according to Hanahan, Turner & Jayko (1951). Phosphatidic acid (PA) was prepared enzymically from PC according to Kates (1956). Phosphatidyl ethanolamine (PEA) and phosphatidyl serine (PS) were prepared from sheep brain by the method of Folch (1942, 1949), based on the differences in solubility of the phospholipids in various mixtures of chloroform and ethanol.

Reaction system. The test mixture was composed of 4–5μmoles of substrate suspended in 0·5 ml. of water and 0·5 ml. of enzyme suspension at pH 6·5. A solution containing 30μmoles of $CaCl_2$ (0·05 ml.) was added to the test mixture, except when PA served as substrate. The incubation was carried out in 10 mm. × 100 mm. test tubes at 37°. It was found that under these conditions the reaction velocity was linear until 80% of the substrate was decomposed.

Measurement of enzyme activity. The enzyme activity was measured by the decrease in concentration of acyl ester bonds after a period of incubation by the following procedure: The reaction was stopped by the addition of 4 ml. of ethanol–diethyl ether (3:1, v/v). The tubes were centrifuged for 3 min. at 1000 *g*. Samples of the supernatant (2 ml.) were transferred into 15 mm. × 150 mm. tubes and the volume was brought to 3 ml. by the addition of the ethanol–ether mixture and acyl ester-bond concentration was estimated by the hydroxamic acid method of Stern & Shapiro (1953). A unit of enzyme activity was defined as the amount of enzyme catalysing the splitting of μequiv. of ester bonds in 1 hr. at 37°. Specific activity was expressed in units/mg. of protein N.

Preparation of enzyme

Step 1: *homogenization of pancreas.* Ox pancreas, which had been stored at −12° for at least 2 weeks, was used as a source of the enzyme. Such pancreas showed appreciable activity, which was preserved for many months if the tissue was kept frozen. Samples of the frozen pancreas were thawed and homogenized with twice their weight of water in a Waring Blendor for about 1 min. The homogenate was filtered by squeezing through a double layer of gauze. The filtrate is referred to as enzyme preparation I.

Step 2: *separation from soluble proteins.* Preparation I was centrifuged for 1 hr. at 20 000 *g* at 3–6° and the turbid supernatant layer discarded. (With pancreas preparations which had been kept frozen for more than a month, longer centrifuging was needed.) The precipitate was resuspended by blending in the original volume of 0·05 M-sodium ethylenediaminetetra-acetate solution, pH 6·0, and centrifuged for 10 min. as before. The slightly turbid supernatant layer was again discarded and the precipitate resuspended in water and again recovered by centrifuging. The supernatant layer, which was usually practically clear, was discarded. The precipitate was again resuspended with the aid of a Waring Blendor in 0·067 M-Sørensen phosphate buffer, pH 6·5. This is referred to as enzyme preparation II.

* Part of a Ph.D. thesis to be submitted to the Senate of the Hebrew University, Jerusalem, Israel.

Table 1. *Substrate specificity and degree of purification of phospholipase A preparations from pancreas*

Activity was measured as described in the Methods section with lecithin as substrate. (A unit of enzyme action was defined as the amount of enzyme catalysing the splitting of 1 μequiv. of ester bonds in 1 hr. at 37°.) Preparation I: crude homogenate; II: washed particles from the homogenate; III: heat-treated particles; IV: purified heat-treated particles.

Enzyme preparation	Total activity (units)	Specific activity (units/mg. of protein N)	Substrates decomposed by the preparation
I	156	0·02	PC; PA; lysolecithin; triglycerides; PEA; PS
II	84	0·15	PC; PA; lysolecithin; triglycerides; PEA; PS
III	63	0·26	PC; PA; PEA; PS
IV	21	0·87	PC; PA; PEA; PS

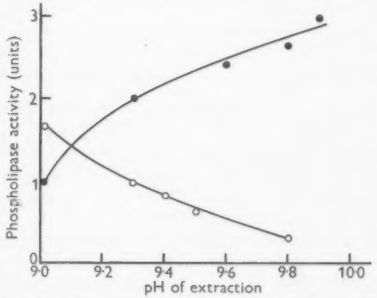

Fig. 1. Solubility of pancreatic phospholipase A in glycine buffer of various pH values. Enzyme preparation III (see Table 1 and text) was suspended in 0·1 M-glycine–NaOH buffer, of various pH values, at room temp. The suspension was centrifuged for 10 min. at 3–6° at 20 000 *g* and phospholipase A activity was measured in the supernatant (●) and in the precipitate, which had been readjusted to pH 6·5 (○), by the method described with lecithin as substrate.

Quantitatively it contained particles from 1 g. of pancreas in 2 ml. of suspension.

Step 3: *destruction of phospholipase B activity.* Enzyme preparation II was heated at 70–75° for 10 min., cooled under tap water and centrifuged for 15 min. at 20 000 *g* at 3–6°. This treatment destroyed any phospholipase B activity present, and the activity of phospholipase A remained unchanged. The precipitate is referred to as preparation III.

Step 4: *dissolving and reprecipitating.* The solubility of the enzyme at various pH values in glycine–NaOH buffer is shown in Fig. 1. When dissolved at pH 9·8, addition of ethanol up to 32% (v/v) did not bring about precipitation of the enzyme, and higher concentrations destroyed it. Accordingly, preparation III was resuspended at room temperature in 0·1 M-glycine–NaOH buffer, pH 9·8, which contained 32% (v/v) of ethanol. The undissolved material was removed by centrifuging for 10 min. at 13 000 *g* at 3–6°. The supernatant was adjusted to pH 6·5–7·0 with N-HCl. The precipitate that formed was collected by centrifuging for 20 min. at 20 000 *g* at 3–6° and resuspended in half the original volume of 0·067 M-phosphate buffer, pH 6·5. This is referred to as enzyme preparation IV and contained, in 1 ml. of suspension, particles originating from 1 g. of pancreas. The specificity, state of purity and the yield of the four enzyme preparations is given in Table 1. It is evident that after the third step in the preparation only phospholipase A activity is present and that the four steps described yield at least a 40-fold purification, since the activities for preparations I and II given are too high, owing to the lysolecithinase activity which still prevails in them.

Table 2. *Influence of calcium ions on the enzymic decomposition of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine and phosphatidic acid by pancreatic phospholipase A*

Enzyme preparation II (see Table 1 and text) was used in these experiments with the indicated concentrations of Ca^{2+} ions and 4 μmoles of substrates. Time of incubation with PC was 60 min., with PA 30 min. and with PEA and PS 5 min.

Concn. of Ca^{2+} ions present (M)	Enzymic decomposition (μmoles) of			
	PC	PEA	PS	PA
0	0·0	0·0	0·0	2·5
0·02	2·9	1·2	1·5	—
0·04	3·1	1·0	1·7	0·5

RESULTS

Influence of calcium ions upon enzyme activity. Previous observations, that the presence of Ca^{2+} ions is required for phospholipase A activity (Zeller, 1950; Hayaishi & Kornberg, 1954), could be verified for the action of the enzyme on PC, PEA or PS. On the other hand, when PA served as a substrate, Ca^{2+} ions had an inhibitory effect. Table 2 shows the influence of Ca^{2+} ions on the enzymic decomposition of PC, PEA, PS and PA.

Heat-stability of the enzyme. Various figures are given in the literature for the heat-stability of phospholipase A from various sources. A bacterial preparation was stable to 97° at pH 9 for 5 min. (Hayaishi & Kornberg, 1954). A phospholipase A from snake venom was stable up to 97° at pH 5·9 (Hughes, 1935). An enzyme extracted from pancreatin was 80% destroyed by heating it to 70° for 10 min. at pH 6·8 (Hanahan, 1952).

It was found that our enzyme preparations were stable up to about 80° when heated for 10 min. at

pH 6·5. When the temperature was raised above this point, inactivation started. However, the temperatures producing inactivation differed from preparation to preparation. This may be partly due to the amounts of Ca^{2+} ions retained in the preparation, since addition of Ca^{2+} ions lowered the temperature at which inactivation started. When 0·02 M-Ca^{2+} ion was present during heating, temperatures between 70° and 90° caused inactivation when PC was subsequently used as the substrate, but not with PA, PEA or PS (Table 3).

Reactivation of the heat-inactivated enzyme. Enzymic preparations which had lost their activities towards PC through heating for 10 min. at 90° in the presence of Ca^{2+} ions, could be reactivated by pre-incubation at 37° for 2–4 hr. (Fig. 2). The presence of PC during the reactivation period did not influence the process of reactivation. Owing to this reactivation effect, complete inactivation could be demonstrated only when the incubation time of the tests was short (30–60 min.). With longer incubations, heat-inactivation became less and less pronounced.

Product of decomposition of phosphatidic acid. As PA has previously not been used as a substrate of phospholipase A, the identification of the reaction product was undertaken. In the reaction at 37° after 2 hr. the acyl ester-bond concentration was halved. No further decrease was observed when the incubation was continued for another 20 hr. Thus the reaction product was a monoacyl compound derived from PA by hydrolysis of one of the two fatty acids attached to the glycerol moiety.

The isolation of the product was based on its solubility properties, which resembled those of PA except that the sodium salt was insoluble in ether and gave a clear solution in water at 37°. The procedure used for isolation of the product was as follows: 100 mg. of PA was suspended in 25 ml. of water; to this was added 50 ml. of enzyme preparation III and the mixture was incubated for 3 hr. at 37°. The reaction was stopped by the addition of 15 ml. of N-perchloric acid, and the whole was then extracted with ether (3 × 100 ml.). The combined ether extracts were concentrated *in vacuo* to the minimum volume which permitted a clear solution of the lipids therein, and 4 vol. of ethanol were added. Any precipitate that formed was discarded. To the clear ethanol–ether solution 40 % (w/v) NaOH soln. was added in drops until

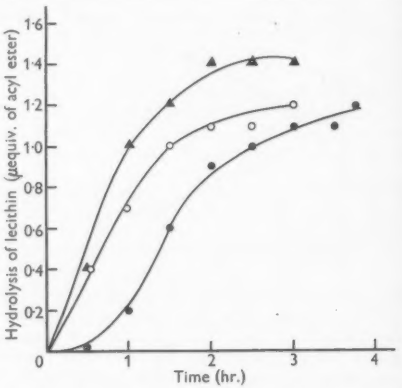

Fig. 2. Reactivation of heat-inactivated phospholipase A. Enzyme preparation II (see Table 1 and text) was heated for 10 min. at 90° in the presence of 0·02 M-Ca^{2+} ions. Its activity was determined by incubation with PC as described. ▲, Unheated enzyme; ●, heated enzyme; ○, enzyme which had been pre-incubated for 4 hr. at 37° after heating.

Table 3. *Decomposition of phosphatidyl choline, phosphatidic acid, phosphatidyl ethanolamine and phosphatidyl serine by heat-treated phospholipase A*

Preparation II (see Table 1 and text) was used as the enzyme. The preparation was heated for 10 min. at 90° at pH 6·5 in the presence of 0·02 M-Ca^{2+} ions. PC and PA were incubated at pH 6·5; PEA and PS at pH 7·0. Phospholipase A activity was measured as described in the text.

		Hydrolysis of μequiv. of ester upon incubation with							
		Untreated enzyme				Heated enzyme			
		Time (min.)							
Expt.	Substrate	10	15	30	60	10	15	30	60
1	PC	—	—	0·7	1·4	—	—	0·2	0·6
	PA	—	—	1·0	1·4	—	—	0·9	1·1
	PEA	1·1	1·3	—	—	1·1	1·3	—	—
	PS	0·6	0·7	—	—	0·6	0·7	—	—
2	PC	—	—	—	1·0	—	—	—	0·0
	PA	—	—	1·5	—	—	—	1·5	—
	PC*	—	—	—	1·3	—	—	—	0·9
	PA*	—	—	1·2	—	—	—	0·9	—

* Ca^{2+} ions omitted.

a pH value of 6·0–6·5 was obtained. The precipitate which formed was collected by centrifuging and extracted with ether (2 × 10 ml.). The ether extracts were discarded. The ether-washed precipitate was dried and dissolved in water at 37°. Upon analysis it contained phosphorus and acyl ester bonds in a molar ratio of 1:1, and 90–95 % of the theoretical values calculated as a disodium salt of monoacylglycerophosphate. Its structure was established according to the method of Hanahan (1954) by oxidation with potassium permanganate followed by acid hydrolysis and analysis of organic and inorganic phosphate. According to this method, the structure was of a β-acyl-α-glycerophosphate. The product was found to have haemolytic properties of a potency equalling that of lysolecithin (Rimon, 1958).

DISCUSSION

Lecithinase A was found to be inactive in fresh ox pancreas and to become activated during storage in the frozen state. The mechanism of this activation is not yet clear. Experiments conducted with the aim of activating the enzyme by autolysis at higher temperatures gave variable results. This may perhaps explain the variations in activities found by various authors working with different commercial-pancreatin preparations.

The enzyme, which is soluble at neutral pH values, was much more active towards phosphatidyl serine, phosphatidyl ethanolamine and phosphatidic acid than towards phosphatidyl choline. The choline group seems to be detrimental to the enzymic cleavage. The difference between the substrates is even more pronounced with the heat-treated enzyme. Heating to 90° in the presence of Ca^{2+} ions had almost no effect on the activity towards all the non-choline substrates but destroyed activity towards lecithin. Experiments to elucidate these special properties of the enzyme towards phosphatidyl choline are in progress.

The product formed by the action of phospholipase A on phosphatidic acid, β-acyl-α-glycerophosphate, has, as far as we are aware, not previously been isolated as the product of an enzymic reaction. Other monoacylglycerophosphate compounds have been prepared synthetically; thus Arnold (1938) synthesized α-acyl-α′-glycerophosphate and α-acyl-β-glycerophosphate, and Kabashima (1938) prepared α-palmitoyl-α′-glycerophosphate. It will be of interest to see whether these isomers resemble our product in haemolytic properties and what structural features are required for haemolysis.

SUMMARY

1. The preparation and purification of phospholipase A from aged ox pancreas is described. No phospholipase A activity could be demonstrated in fresh ox pancreas.

2. The enzyme preparation was insoluble at neutral pH and hydrolysed phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine and phosphatidic acid.

3. The rate of reaction was much slower with phosphatidyl choline than with the other substrates.

4. The product of the enzymic decomposition of phosphatidic acid was isolated and identified as β-acyl-α-glycerophosphate.

5. Ca^{2+} ions were required for the enzymic decomposition of all the substrates except phosphatidic acid.

6. By heating the enzyme preparation to 70–90° in the presence of Ca^{2+} ions the activity towards phosphatidyl choline was lost, whereas that with the other substrates remained unimpaired. The heat-inactivated enzyme could be reactivated by pre-incubation at 37° for several hours.

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Rates of Oxidation of Galactose and Glucose in Erythrocytes and Liver after Lactose Feeding

By ANUBHA CHOWDHURY and D. P. SADHU
Department of Physiology, University College of Science and Technology, Calcutta and Department of Physiology and Nutrition, Bengal Veterinary College, Belgachia, Calcutta

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Fiegelson & Conte (1954) could find no adaptive increase in galactose oxidation in adult rats fed on a galactose diet. Young rats grew better when fed on 30% of dry matter of the diet as lactose (Whittier, Cary & Ellis, 1935; Chowdhury & Sadhu, 1958), while high lactose or galactose feeding is toxic to rats (Ershoff & Deuel, 1944; Guha, 1931). These studies and those in galactosaemia (Handler, 1947; Schwarz, Goldberg, Konrower & Holzal, 1956) led us to study the rates of oxidation of galactose and glucose in erythrocytes and liver tissue in young rats fed on a lactose diet, since it was thought that the young animals were likely to show better adaptive formation of enzymes for galactose oxidation.

EXPERIMENTAL

Series 1. Albino rats of the Bengal Veterinary College stock weighing about 30 g. were divided into two groups and fed *ad lib.* Group 1 was kept on a diet containing 70% of sucrose, 15% of casein, 5% of cod-liver oil, 5% of coconut oil, 4% of salt mixture (Hubbell, Mendel & Wakeman, 1937) and 1% of yeast. In group 2, instead of 70% of sucrose, 50% of sucrose + 20% of lactose were given, the other constituents of the diet being the same as in group 1. Direct measurement of glucose and galactose oxidation in erythrocytes was carried out by the procedure of Fiegelson & Conte (1954) with a few modifications. The animal was anaesthetized with ether–chloroform, the chest was opened and blood was collected into a heparinized syringe directly from the heart. The blood was centrifuged at 2500 rev./min. at 5–10° for 10 min. The erythrocytes were suspended in 3 vol. of ice-cold Krebs–Ringer phosphate buffer (pH 7·4) (Umbreit, Burris & Stauffer, 1957) and centrifuged at 2500 rev./min. at 5–10° for 10 min. This washing was repeated twice and finally the packed erythrocytes (centrifuged at 2500 rev./min. for 10 min.) were suspended in 2 vol. of ice-cold Krebs–Ringer phosphate buffer (pH 7·4).

A similar method was applied to study liver galactose-oxidizing activity; 10% liver homogenate in Krebs–Ringer phosphate buffer (pH 7·4) was used. A portion of the right lobe of the liver was placed on a watch glass put on cracked ice; it was dried by filter paper, weighed in a torsion balance and quickly transferred to Krebs–Ringer phosphate buffer (pH 7·4) in the homogenizer tube, which was placed in a beaker containing cracked ice and the tissue homogenized while the tube was in ice.

In the main vessel of the Warburg flask were placed 1 ml. of erythrocyte suspension, 0·9 ml. of Krebs–Ringer phosphate (pH 7·4), 0·3 ml. of mM-adenosine triphosphate (ATP) solution, 0·2 ml. of 0·05% methylene blue or (for liver) 1 ml. of homogenate, 1 ml. of Krebs–Ringer phosphate (pH 7·4) and 0·4 ml. of mM-ATP solution. In the side arm was placed 0·5 ml. of 33 mM-glucose or galactose or 0·5 ml. of Krebs–Ringer phosphate (pH 7·4) for the blank; the central cup contained 0·1 ml. of 30% potassium hydroxide solution with 1 cm.² filter paper. Oxygen consumption was measured for 1 hr. at 38° by tipping over the substrate from the side arm after 10 min. temperature equilibration.

Series 2. Rats weighing 30–35 g. were divided into three groups. Groups 1 and 2 were fed *ad lib.* in the same way as groups 1 and 2 of series 1. Group 3 was fed on a diet similar to that of group 1 except that it contained 40% of lactose and 30% of sucrose. The apparent galactose oxidation by liver in rats of series 1 might have been affected by the erythrocytes contained in the same tissue; therefore in series 2 the liver was first perfused with cold Ringer solution through the hepatic artery till the liver was quite blanched. The nitrogen content of the tissues was determined by the standard micro-Kjeldahl method. Other details were as in series 1.

RESULTS AND DISCUSSION

The results of series 1 are given in Tables 1 and 2. It will be observed from Table 1 that after 20 days there was no difference between the two groups in the rates of oxidation by the erythrocytes. Oxygen consumption with galactose was small. When the feeding period was increased to 45 days, galactose-oxidizing capacity was considerably increased in group 2, i.e. in the lactose-fed group. The rate of oxygen consumption with galactose substrate was more than 50% of that obtained with glucose substrate. The rate of glucose oxidation was about the same in the two groups.

Galactose-oxidizing activity could not be detected in liver homogenate by this method after 1½ months (the reading of the blank was the same as the reading with galactose as substrate). When feeding was continued for 3 months there was a small amount of galactose-oxidizing activity in the lactose-fed group but not in the sucrose-fed control group (Table 2). These results show an increased

Table 1. *Galactose- and glucose-oxidizing activity of erythrocytes after lactose feeding*

Rats were fed on a diet containing *ad lib.* either 70 % of sucrose (group 1) or 50 % of sucrose + 20 % of lactose (group 2). Each figure represents the average of estimation in two rats.

No. of days of feeding (approx.)	Group	Average gain in body wt. (g./week)	O_2 consumed (μl./ml. of packed erythrocytes for 30 min.) With glucose as substrate	With galactose as substrate
20	1	6·2	126	3·7
	1		120	4·2
	1		110	3·6
	2	6·3	129	5·3
	2		98·5	3·3
	2		127·3	3·2
45	1	5·5	122	7·0
	1		115	6·3
	1		120·5	5·6
	1		125	7·5
	2	6·8	109·3	68·3
	2		124	72·8
	2		123·2	71·7
	2		129·3	73·1

Table 2. *Galactose-oxidizing activity of liver after lactose feeding for 3 months*

Each figure represents the average of estimations in two rats. Groups are as in Table 1.

Group	Average gain in body wt. (g./week)	O_2 consumed (μl./ml. of liver homogenate in 30 min.) No substrate	With galactose as substrate
1	4·5	22·6	21·9
1		23·4	23
1		20·1	21
2	5·5	20·9	35·75
2		18·7	26·65
2		20·7	33·6

Table 3. *Galactose- and glucose-oxidizing activity of erythrocytes and of liver after 6 months' lactose feeding*

Groups 1–3, received 0, 20 and 40 % respectively of lactose substituted for sucrose and fed *ad lib.* Q_{O_2} is determined as μl. of O_2/mg. of N/hr. in erythrocytes and as μl. of O_2 consumed/mg. of dry tissue/hr. in liver at 38°.

No. of group	No. of experiments	Average gain in body wt. (g./week)	Tissue	Q_{O_2} with glucose substrate (mean ± S.E.)	t (D.F. 10) compared with group 1	Q_{O_2} with galactose substrate (mean ± S.E.)	t (D.F. 10) compared with group 1
1	6	4·5	Erythrocytes	3·62 ± 0·47	—	0·84 ± 0·04	—
2	6	5·5	Erythrocytes	2·78 ± 0·24	3·8	2·88 ± 0·26	19
3	6	3·2	Erythrocytes	2·40 ± 0·27	5·1	1·35 ± 0·15	8
1	6	4·5	Liver	0·62 ± 0·15	—	0·28 ± 0·11	—
2	6	5·5	Liver	0·42 ± 0·07	4	0·62 ± 0·11	5·3
3	6	3·2	Liver	0·103 ± 0·029	10·5	0·33 ± 0·056	1*

* Difference is not significant.

galactose oxidation in erythrocytes and liver after a prolonged feeding of lactose, such as 1½ months after feeding of lactose in erythrocytes and after 3 months in liver. Table 3 shows the results of a study of glucose and galactose oxidation after feeding rats for six months (series 2). It will be observed from Table 3 that Q_{O_2} value of erythrocytes with galactose substrate was much higher in group 2 than in group 1. In these rats of group 2 the average value of Q_{O_2} with galactose substrate was very near to the Q_{O_2} with glucose substrate. In Group 3, where the diet included 40 % of lactose, the Q_{O_2} value with galactose substrate was less than in group 2, but it was still significantly higher than in group 1.

With liver homogenate similar results were obtained, i.e. galactose oxidation was significantly higher in group 2 (20 % of lactose) than in group 1 (control group), but there was no significant difference between groups 3 and 1. The oxidation rate of liver homogenates was rather low; the use of a homogenate with Ringer-phosphate would not be

expected to give maximum rates of carbohydrate oxidation (Wenner, Dunn & Weinhouse, 1954).

All these results show that an adaptive increase in the oxidation of galactose in erythrocytes and liver takes place in rats when the diet contains 20% of lactose; this increase is lowered when lactose content of the diet is increased to 40%. Galactose oxidation by liver and erythrocytes in group 1 of series 2 was higher than that of group 1 of series 1. This indicated that the galactose-oxidizing capacity increased with age. Glucose oxidation was decreased in erythrocytes and liver of rats receiving 20% of lactose in the diet and such decrease was still greater in erythrocytes of rats receiving 40% of lactose (Table 3).

The increased oxidation of galactose might be due to an increase in enzyme concentrations, permeability effects or alterations in cofactor concentrations. It has not been possible to differentiate between these causes in the present work. The control rats used by Fiegelson & Conte (1954) showed high galactose oxidation and the oxidation rate in these adult rats could not be increased by galactose administration, whereas in our experiments young rats were used where galactose oxidation in the control rats was low and lactose feeding increased the galactose oxidation. The galactose-oxidation capacity of our control rats increased with age, which is in conformity with the initial high galactose-oxidation capacity of the adult rats used by Fiegelson & Conte (1954).

SUMMARY

1. Three groups (1–3) of albino rats (30–35 g.) were placed on diets in which respectively 0, 20 and 40% of lactose was substituted for sucrose.

2. After 45 days' feeding, galactose-oxidizing activity in the erythrocytes had increased considerably in group 2, but not in group 1.

3. After 3 months' feeding, galactose-oxidizing activity of suspensions of liver in Ringer-phosphate had increased in group 2 but not in group 1.

4. After 6 months' feeding the rates of consumption of oxygen (/mg. of N of erythrocytes or /mg. dry wt. of liver) with galactose as substrate had increased in group 2 compared with group 1.

5. In group 3 this increase was less in the erythrocytes and insignificant in liver. The rates of consumption of oxygen by erythrocytes and liver with glucose substrate were lower in group 2 than in group 1, and still lower in group 3.

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Studies on Gastric Proteolysis

4. PROTEINASE ACTIVITY OF GASTRIC JUICE AND GASTRIC-MUCOSAL EXTRACTS AT pH 6 TO 8*

By W. H. TAYLOR
Department of Clinical Biochemistry, Radcliffe Infirmary, University of Oxford

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There have been several reports of the existence in gastric juice and gastric-mucosal extracts of a weak proteinase with maximal activity at neutral pH (Willstätter & Bamann, 1929; Klein & Wilkinson, 1933; Griffiths, 1934; Emerson & Helmer, 1936; Taylor, Castle, Heinle & Adams, 1938; Fox, 1949; Glass & Boyd, 1954; Janowitz & Hollander, 1954). Except for that of Willstätter & Bamann, these reports have been concerned with gastric mucolysine (Glass & Boyd, 1954; Janowitz & Hollander, 1954), an enzyme which liberates tyrosine-containing products from human gastric mucus, or with the intrinsic anti-pernicious anaemia factor of Castle (1929). This factor occurs in gastric juice and

* Part 3: Taylor (1959*c*).

gastric-mucosal extracts, and several attempts have been made to show that its action upon ox muscle at pH 7·0, to yield a product which will cause a remission of pernicious anaemia, is proteolytic. Taylor *et al.* (1938) found a parallel variation between the intrinsic-factor activity of various preparations and their proteolytic activity at neutral pH, and showed that the gastric juice of patients with pernicious anaemia possessed neither action. Since the discovery of vitamin B_{12}, the hypothesis that intrinsic factor is a proteolytic enzyme has been largely abandoned, but the nature of the observed proteolytic activity of gastric juice at neutral pH remains obscure. It is not definitely established whether this activity results from true gastric secretion or whether it arises from traces of pancreatic or duodenal enzymes which have been regurgitated into the stomach, or from saliva, leucocytes or desquamated cells. Even if it results from gastric secretion, the question has been raised whether the activity could not be accounted for by a weak action of pepsin at neutral pH (Jones, Grieve & Wilkinson, 1938). Experiments concerned with these problems are now described.

EXPERIMENTAL

The methods and materials used have been described previously (Taylor, 1957, 1959*a*). Saliva was collected from normal human subjects by simple expectoration without stimulation by chewing. Factor B (Welch & Heinle, 1951) was donated via the Nuffield Department of Clinical Medicine by Professor A. D. Welch. Glandular mucoprotein was prepared by the method of Glass, Boyd, Rubinstein & Svigals (1952).

Enzyme–substrate mixtures were brought to a suitable pH for digestion by addition of 67 mM-disodium hydrogen phosphate–potassium dihydrogen phosphate buffer. The change of pH which occurred during proteolysis was less than 0·1 unit.

RESULTS

Proteolytic activity of human gastric juice

Action on plasma protein. Twenty-two observations were made upon fourteen normal subjects, over the range pH 6·0–8·0 (Table 1). These specimens of gastric juice were free from contamination with duodenal contents, as judged by the criteria described subsequently. Nine subjects showed activity as measured by an increased formol titration, and five did not. Definite peaks of activity were detected in only seven subjects, all of whom showed in one or more samples of gastric juice a maximum at pH 6·8–7·2 (Fig. 1). Three subjects showed activity at pH 7·5–7·7 as well. The amount of proteolysis at the maximum at pH 6·8–7·2 varied from one-tenth to one-quarter of that at the maximum at pH 1·7–2·4, and digestion at the maximum at pH 7·5–7·7 was of the same order.

Action on other proteins. Two samples of gastric juice which digested plasma at neutral pH also digested casein with maxima at pH 6·9 and 7·5 respectively (Table 1). The amount of proteolysis was about one-eighth of that at pH 1·8–2·2. Human serum albumin was slightly digested by gastric juice from two other subjects but no clearly defined peaks of activity could be determined. Egg albumin was not digested at neutral pH by the gastric juice from three subjects which had previously been shown to digest plasma protein. Subjects whose juice failed to digest plasma protein also failed to digest casein and human serum albumin.

Further investigation of inactive juices. Griffiths (1934), Emerson & Helmer (1936), Taylor *et al.* (1938) and Fox (1949) found the proteolytic activity of gastric juice at neutral pH to be much more easily demonstrable by techniques involving the estimation of increase of non-precipitable nitrogen than by those estimating liberated amino or carboxyl groups directly. The failure to detect activity in five of fourteen normal subjects might perhaps be thus explained. The five inactive juices were therefore incubated with plasma protein and with casein. After precipitation of proteins by adding trichloroacetic acid to a final concentration of 5% (w/v), the total N of the filtrate was determined by the micro-Kjeldahl method and compared with that from the usual control tubes, mixed after separate incubation. In no case had significant amounts of digestion occurred.

Proteolytic activity of gastric-mucosal extracts

Extracts of normal human fundic mucosa from two subjects showed slight digestion of plasma protein over the range pH 6·4–7·2 (Table 1). An extract of human pyloric mucosa was inactive.

An extract of dried swine mucosa (Pepsac) in 0·01 N-HCl digested plasma protein with a maximum at pH 6·7 and casein with one at pH 6·4. An extract in 2% NaCl solution digested human serum albumin with a maximum at pH 6·6. An extract of 'Gaster siccata' B.P.Codex in 2% NaCl showed proteolytic activity at pH 6·5–7·2 upon plasma protein and casein, although a significant maximum could not be established. This extract was, however, inactive on egg albumin.

Commercial rennet (a NaCl–glycerol extract of calf gastric mucosa) digested plasma protein with a pH maximum in the range pH 7·0–7·2 (Table 1) but failed to digest egg albumin.

Table 1. *Proteinase activity of human gastric juice and of human, swine and calf gastric-mucosal extracts over the range* pH 6·0–8·0

The amount of liberated amino acid nitrogen in this table and in the accompanying figures is that in the whole digest, which consisted usually of 3 ml. of buffer, 1 ml. of gastric juice or enzyme extract and 1 ml. of plasma or 5% (w/v) human serum albumin or 5% (w/v) egg albumin or 2 ml. of 2% (w/v) casein. Temp. 37°; 3 hr. 'Insulin juice' and 'histamine juice' refer to the gastric secretions obtained after intravenous injection of insulin and subcutaneous injection of histamine respectively.

Subject or extract	Substrate	Proteinase activity: Maximum pH	Proteinase activity: mg. of amino acid N
Human			
1. Resting juice	Plasma	6·9	0·18
2. Resting juice	Plasma	Active, no maximum	
Resting juice	Plasma	6·8	0·18
Insulin juice	Plasma	7·5	0·18
3. Insulin juice	Plasma	Active, no maximum	
4. Resting juice	Plasma	6·9	0·14
		7·5	0·12
Resting juice	Plasma	7·5	0·30
Insulin juice	Plasma	Active, no maximum	
Insulin juice	Plasma	Active, no maximum	
Insulin juice	Plasma	7·7	0·20
Insulin juice	Plasma	7·6	0·15
5. Resting juice	Plasma	Inactive	
6. Histamine juice	Plasma	Active, no maximum	
7. Histamine juice	Plasma	Inactive	
8. Histamine juice	Plasma	Inactive	
9. Histamine juice	Plasma	7·1	0·06
10. Histamine juice	Plasma	7·1	0·09
11. Histamine juice	Plasma	Inactive	
12. Histamine juice	Plasma	Inactive	
Histamine juice	Plasma	Inactive	
13. Histamine juice	Plasma	6·9	0·08
		7·6	0·06
14. Histamine juice	Plasma	7·2	0·09
4. Insulin juice	Casein	7·5	0·04
10. Histamine juice	Casein	6·9	0·08
6. Histamine juice	Human serum albumin	Active, no maximum	
9. Histamine juice	Human serum albumin	Active, no maximum	
1. Resting juice	Egg albumin	Inactive	
9. Histamine juice	Egg albumin	Inactive	
13. Histamine juice	Egg albumin	Inactive	
Fundic mucosal extract 1	Plasma	Active, no maximum	
Fundic mucosal extract 2	Plasma	Active, no maximum	
Pyloric mucosal extract 1	Plasma	Inactive	
Swine			
Pepsac (0·01 N-HCl extract)	Plasma	6·7	0·09
Pepsac (0·01 N-HCl extract)	Casein	6·4	0·05
Pepsac (2% NaCl extract)	Human serum albumin	6·6	0·12
Gaster siccata (2% NaCl extract)	Plasma	Active, no maximum	
Gaster siccata (2% NaCl extract)	Casein	Active, no maximum	
Gaster siccata (2% NaCl extract)	Egg albumin	Inactive	
Calf			
Commercial rennet 1	Plasma	7·0	0·06
Commercial rennet 2	Plasma	7·3	0·05
Commercial rennet 1	Egg albumin	Inactive	

Paper chromatography of digestion products

Table 2 shows that digests of plasma protein and gastric juice at pH 6·9 contain products staining with ninhydrin which were not present before digestion. This confirms that proteolysis has in fact taken place.

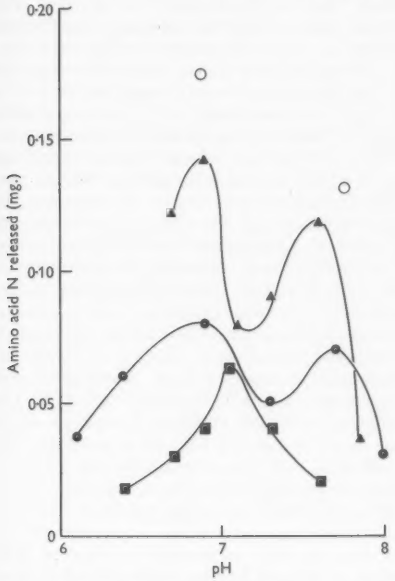

Fig. 1. pH–Activity curves for the digestion of plasma protein at pH 6·0–8·0 by gastric juice from three normal subjects (■, ●, ▲). Digestion mixtures consisted of 3 ml. of buffer, 1 ml. of gastric juice and 1 ml. of plasma. The isolated points (○) were obtained by doubling the concentrations of gastric juice (●) and substrate in the digestion mixture. Temp. 37°; 3 hr.

Table 2. *Paper chromatography of digestion products from two samples of normal human gastric juice incubated with plasma protein*

Temp. 37°, for 2 hr. at pH 6·9. 78% (w/w) Phenol in water. Ninhydrin stain.

Gastric juice A		Gastric juice B	
R_F	Amount of product	R_F	Amount of product
0·06	+	—	–
0·17	+ + +	0·17	+ +
0·25	+	0·25	+
0·33	+	—	–
0·42	+	—	–
0·54	+ +	0·54	+
0·77	+	—	–

Investigation of possible sources of error

Effect of gastric urease. This enzyme is known to act over the range pH 6·0–8·0. If ammonia were liberated by it during the present experiments, a spuriously high formol-titration would result from the subsequent interaction of formaldehyde and NH_4^+ ions. Inaccuracy in the determination of pH maxima might thus arise. Direct determination of ammonia in the digestion mixtures with Nessler's reagent revealed, however, that there was no excess liberation of ammonia above the small amounts occasionally found in controls. This is probably because gastric urease is rapidly inactivated (Martin, 1933; Hollan, 1947) by the high H^+-ion concentrations at which the juices were secreted (pH 2·0) and stored (pH 3·0–4·0). Experiments with unacidified resting gastric juice showed that gastric urease exhibits a pH maximum at pH 6·6 but that, at the low substrate concentrations likely to be present in digests containing plasma, the amount of ammonia formed even under these favourable conditions would not significantly affect the proteolytic pH–activity curves.

Effect of duodenal contents. The duodenum may contain trypsin, chymotrypsin and several peptidases, all of which exert maximal proteolytic activity in the range pH 6·0–8·0. If its contents were to be regurgitated into the stomach in the absence of bile, contamination would occur which could not be spotted by the naked eye.

In Fig. 2 are compared the pH–activity curves upon plasma of a colourless and a heavily bile-stained gastric juice obtained during the same histamine test meal in a normal subject. The colourless juice has a plateau of activity from pH 6·2 to 7·4 and virtually no activity at pH 7·8, whereas the contaminated juice has an optimum at pH 7·8. Subtraction of the former curve from the latter, on the assumption that activity equal to that of the former curve is present in the latter, yields a curve with a maximum at pH 7·9. This may be regarded, approximately, as the difference in proteolytic activity brought about by contamination with bile-stained duodenal contents. This curve is in many respects similar to that of Fig. 3, in which bovine trypsin is digesting plasma with only slight activity at pH 6·0 and with a maximum at pH 7·9. Colourless juices which gave activity in the region pH 7·5–8·0 were therefore regarded with suspicion, lest there had been a regurgitation of non-bile-stained duodenal materials. In such juices the hydrolytic action upon chloracetyl-L-tyrosine, which is a specific substrate for pancreatic carboxypolypeptidase, was investigated. Any juice in which this enzyme was thus detected was considered to be contaminated with duodenal contents and its proteolytic activity at neutral pH was

ignored. The absence of bile, the absence of hydrolysis of chloracetyl-L-tyrosine and the absence of proteolytic maxima at pH 7·9 were thus the criteria upon which freedom from contamination with duodenal material was established. The pH maxima at pH 7·5–7·7 shown in Table 1 are considered not to be of duodenal origin in so far as all these criteria are obeyed.

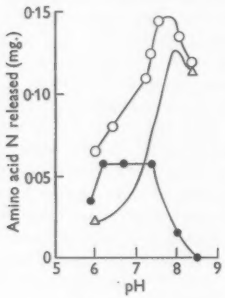

Fig. 2

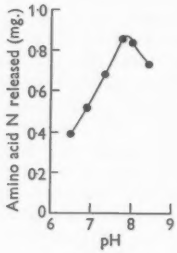

Fig. 3

Fig. 2. pH–Activity curves for the digestion of plasma protein at pH 6·0–8·5 by clear (●) and bile-stained (○) juices from the same normal subject. A curve representing the difference between the two curves is also given (△). Temp. 37°; 3 hr. Digestion mixtures were as described for Fig. 1.

Fig. 3. pH–Activity curve for the digestion by bovine trypsin of plasma protein. Temp. 37°; 3 hr. Digestion mixtures consisted of 5 ml. of buffer, 1 ml. of crystalline trypsin solution (10 mg./100 ml.) and 1 ml. of plasma.

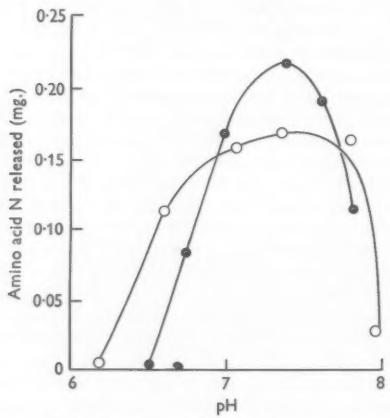

Fig. 4. pH–Activity curves for the digestion of plasma protein at pH 6·0–8·0 by saliva from two normal subjects. Temp. 37°; 3 hr. Digestion mixtures consisted of 2 ml. of saliva, 1 ml. of plasma and 5 ml. of buffer.

Effect of saliva. Saliva is an inevitable contaminant of most samples of gastric juice. When saliva and plasma protein are incubated together, an increase of formol titration occurs. This has been demonstrated in each of five subjects and is maximal at about pH 7·5 (Fig. 4). Upon centrifuging saliva it is found that activity is present in both supernatant and residue (Table 3). It would appear that approximately a quarter of the activity was removed in the centrifuged deposit, the volume of which is about one-hundredth of that of the original saliva. Since the activity of both the supernatant and the salivary deposit has the same pH maximum, it is possible that the proteolytic action of saliva is really that of broken-down cells from the buccal mucosa rather than that of a true enzyme from salivary glands.

Further information about the contaminating effects of saliva may be obtained by investigating the activity of gastric juice or of mucosal extracts under circumstances where the presence of saliva is rigorously excluded. Swine pyloric juice, from an isolated pouch, was found to exert proteolytic activity at neutral pH with a maximum at pH 7·2. Commercial rennet and extracts of human and swine gastric mucosa were maximally active between pH 6·4 and 7·3 but not at higher pH. These results indicate that although proteolytic activity may occur above pH 7·3, when a maximum is found above this limit it is usually with material in which the presence of saliva cannot be excluded.

Effect of bacteria, leucocytes and desquamated cells. The possibility that these contaminants may contribute to the proteolytic activity of gastric juice at neutral pH has been investigated by centrifuging samples of active juice and determining the activity of the precipitate. In each of five subjects it was inactive at pH 6·8–7·2. Even if the bulk of the activity had escaped into the gastric juice, some residual activity of the contaminants might have been expected in the precipitate. These observations would support the view that the enzyme causing proteolytic activity at neutral pH is secreted by the mucous membrane. The possibility that it is derived from bacteria proliferating

Table 3. *Liberation of titratable amino groups from 1 ml. of plasma*

Plasma was in 67 mM-phosphate buffer, pH 7·5. Temp. 37°; 3 hr.

Enzyme preparation	Formol titration (ml. of 0·04N-NaOH)
Saliva (1 ml.)	0·21
Centrifuged supernatant (1 ml.) of saliva	0·15
Centrifuged deposit (1 ml.), dissolved in half original volume	0·10

in the deeper layers of the mucosa, as appears to be the case with gastric urease (Davies & Kornberg, 1955), cannot be excluded.

Effect of pepsin at neutral pH

The evidence that has been presented indicates that gastric juice is sometimes capable of proteolytic action at neutral pH when care is taken to exclude, or allow for, urease action, and for contamination by duodenal contents, saliva, bacteria, leucocytes and desquamated cells. A possible explanation of this proteolytic activity may be that it is caused by pepsin. The activity is not, however, a simple prolongation of that of pepsin at pH 4·0, for it arises as a distinct maximum, and a different pepsin–substrate union from those already considered (Taylor, 1959*b*) would be implied.

Attempts to show that pepsin exerts a proteolytic action at neutral pH have been made by incubating crystalline swine pepsin, pepsin A and electrophoretically separated human pepsins with plasma protein, casein and egg albumin. In no instance was activity between pH 6·0 and 8·0 detected. The fact that some gastric juices of Table 1 have marked peptic activity below pH 5·0, yet do not show activity at pH 6·0–8·0, is further evidence against pepsin being the responsible enzyme, as is the observation that sometimes patients with pernicious anaemia and chronic hypochromic anaemia secrete gastric juice with no peptic activity but with proteolytic activity at neutral pH (Taylor, 1956).

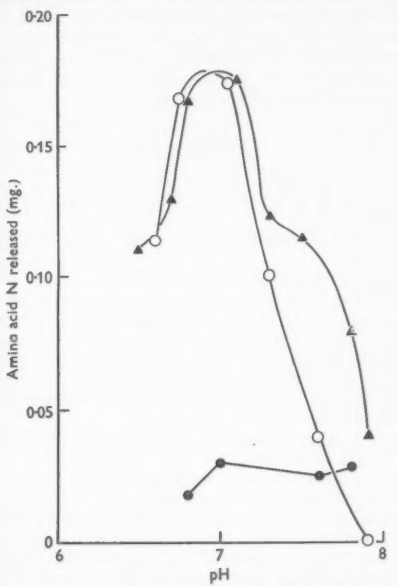

Fig. 5. Effect of ammonium sulphate fractionation upon the proteolytic activity at pH 6·0–8·0 of normal human gastric juice (▲). ○, Precipitate from 50 to 100% saturation, dialysed free of ammonium sulphate and redissolved in half the original volume; ●, supernatant after 100% saturation, similarly dialysed. Plasma protein as substrate. Temp. 37°; 3 hr. Digestion mixtures in each instance consisted of 1 ml. of gastric juice or enzyme extract, 1 ml. of plasma and 3 ml. of buffer.

Further properties of the enzyme responsible for activity at neutral pH

The isolation of a specific substance with activity at neutral pH in solid or crystalline form has not been achieved, but the data of Fig. 5 indicate that the activity may be removed more or less completely from gastric juice by 50–100% saturation with ammonium sulphate. Attempts at further purification have been unsuccessful, as there is considerable loss of activity when subfractionation procedures are attempted. The activity has been found to be stable at neutral pH on storage, as long as the gastric juice remains free from bacterial contamination. Activity is, however, lost when gastric juice is stored for any appreciable length of time (e.g. 1 week) at below pH 3·0 at 0–5°. These properties provide further evidence that the activity at neutral pH is distinct from that of pepsin, which is destroyed at alkaline pH but not below pH 3·0.

Relationship to intrinsic factor

Three active preparations of intrinsic factor have been investigated for proteinase activity over the range pH 6·0–8·0. Of these factor B (Welch & Heinle, 1951) showed activity, but glandular mucoprotein (Glass *et al.* 1952) and substance E (Taylor, 1955; O'Brien, Taylor, Turnbull & Witts, 1955) possessed none.

DISCUSSION

The experiments described above show that the gastric juice of a majority of normal human subjects, and human and swine gastric-mucosal extracts, possess a weak proteinase with maximal activity at pH 6·8–7·2. The responsible enzyme is precipitated by 50–100% saturation with ammonium sulphate. Proteinase activity with a pH maximum at pH 7·5–7·7 is also sometimes present in gastric juice, but not in mucosal extracts. It is probably caused by contamination with saliva, which contains, particularly in the centrifuged deposit, a proteinase which is active at this pH.

The experiments also indicate the caution with which any claim that gastric juice exerts a proteolytic action at neutral pH should be received. It is suggested that such a claim is valid only (1) if

there is an increase of titrable amino or carboxyl groups, accompanied by (2) qualitative evidence of ninhydrin-reactive products on paper chromatograms of the digest, (3) if these occur in the absence of bile and saliva, and of trypsin, chymotrypsin or carboxypeptidase activity, as tested for by absence of hydrolysis of a substrate specific for one of these enzymes, and (4) if separate controls of substrate and enzyme are incubated for the same length of time as the digest (Taylor, 1959*a*). Controls with boiled enzyme, or the reaction mixture at zero time, do not correct for the error of substrate or enzyme autodigestion. When working with small amounts of activity or with sensitive methods of analysis, such as paper chromatography, neglect of this precaution may give a spurious impression of activity.

If these criteria are adopted, much of the previous work on the proteinase activity at neutral pH may be of doubtful significance. The only pH–activity curves for the enzyme that have been published previously (Taylor *et al.* 1938) show an optimum in the region pH 7·5–8·0. The present observations indicate that this activity was caused by duodenal or salivary contamination rather than by the true gastric enzyme.

The previous evidence that gastric-proteinase activity at neutral pH and intrinsic-factor activity are associated depended mainly on the similar behaviour of the two activities to various procedures of fractionation and adsorption. In both glandular mucoprotein (Glass *et al.* 1952) and substance E (O'Brien *et al.* 1955) the two activities are dissociated. It does seem therefore that the two activities are caused by different molecules or occur at different sites on the same molecule. In the latter case, loss of proteinase activity would be explained by fragmentation of the molecule or by inactivation at one site.

The physiological role of the enzyme would seem, on the available evidence, to be unimportant, for under normal circumstances the pH in the stomach when food is present is much more acid than that at which activity of the enzyme *in vitro* can be demonstrated, and even at favourable pH this activity is slight. The apparent absence of the enzyme without obvious ill-effect in about one-third of the subjects who have been investigated would also suggest that its physiological role is slight, as well as perhaps explaining why some earlier workers have doubted its presence. The possibility that the enzyme arises not from gastric cells, but from bacteria proliferating in the deeper layers of the mucosa, remains to be investigated.

SUMMARY

1. Slight proteinase activity, with a pH maximum in the range pH 6·8–7·2, has been found in the gastric juice of nine out of fourteen normal subjects.

2. Similar activity occurred with extracts of normal human, swine and calf gastric mucosa and with swine pyloric juice.

3. This activity was present when contamination with saliva or duodenal contents was excluded and was not concentrated in the desquamated cells and leucocytes of the centrifuged deposit. It did not result from a slight perpetuation at neutral pH of the action of pepsin.

4. The responsible enzyme is precipitated from gastric juice at about pH 5·0 by 50–100% saturation with ammonium sulphate. Castle's intrinsic factor may be concentrated similarly but the two substances are different.

5. Saliva, from five normal subjects, possessed weak proteinase activity with a pH maximum near pH 7·5. This activity is concentrated in the centrifuged deposit which contains mainly buccal squamous cells.

I wish to thank Mr Brian Collett and Miss Barbara Hunt for skilful assistance in part of this work, many colleagues and students for giving gastric juice and saliva, Dr N. G. Heatley for a supply of pig pyloric juice and Dr R. B. Fisher and Mr J. R. P. O'Brien for helpful discussion and for reading and criticizing the D.M. thesis from which the material of this paper is abridged.

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The Effect of Insulin Antibodies on Glucose Uptake by the Isolated Rat Diaphragm

By P. H. WRIGHT
Department of Chemical Pathology, Guy's Hospital Medical School, London, S.E. 1

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During insulin therapy generalized allergic reactions are sometimes observed in diabetic subjects and disappear in many cases when the insulin preparation is changed (see Joslin, Root, White & Marble, 1952). These allergic manifestations are often the result of sensitization to impurities in the insulin preparations (Jorpes, 1949). It is also known, however, that insulin itself is antigenic and that antibodies appear in the plasma of human subjects (Berson, Yalow, Bauman, Rothschild & Newerly, 1956) and of animals (Moloney & Coval, 1955) after only short courses of insulin therapy; no such antibodies are demonstrable in human or animal subjects who have not received parenteral insulin therapy. Serum from insulin-treated human subjects and animals (i) neutralizes the convulsive effect of insulin in mice (Banting, Franks & Cairns, 1938; Lowell, 1942; Sehon, Kaye, McGarry & Rose, 1955; Moloney & Coval, 1955); (ii) binds added ^{131}I-labelled insulin in the γ-globulin (Burrows, Peters & Lowell, 1957) or between the β- and γ-globulin (Berson & Yalow, 1957) fractions of the plasma proteins; (iii) agglutinates or haemolyses red cells conjugated with insulin (Arquilla & Stavitsky, 1956*a*).

In the present investigations serum from guinea pigs sensitized to purified bovine insulin by the method described by Moloney & Coval (1955) was examined to determine its ability to inhibit the effects of insulin and other hypoglycaemic agents upon the isolated rat diaphragm.

MATERIALS AND METHODS

Insulin. Insulin samples from four sources were used (potency stated in international units): (*a*) bovine, six times recrystallized (22·2 units/mg.); (*b*) pig, recrystallized once (approx. 20 units/mg.); (*c*) sheep, recrystallized once (approx. 20 units/mg.); (*d*) human, crude (approx. 1 unit/mg.); this specimen was extracted from human pancreases removed at autopsy 12–15 hr. after death. No gross evidence of disease was visible in these glands, which were removed from subjects who had not suffered from diabetes.

Antigen. Recrystallized bovine insulin (10 mg.) was dissolved in 10 ml. of aq. 0·6% phenol acidified with hydrochloric acid to pH 2·6 (approx.) and emulsified in a Waring Blendor with a mixture of 7 ml. of liquid paraffin (B.P.) and 3 ml. of lanolin (Adeps Lanae, anhydrous, B.P.).

Sensitization of guinea pigs. Each guinea pig, of either sex, was injected subcutaneously with 2 ml. of freshly prepared antigen, 1 ml. being injected between the shoulders and 0·5 ml. into the inner aspect of each thigh. The same dose of antigen was injected 1 month later, the animal being bled by cardiac puncture 2 weeks after the second injection. Subsequent specimens of serum were in some cases obtained from these animals 2 weeks after a further similar course of antigen injections. Groups of four animals were sensitized simultaneously, the serum from animals in each group being combined and stored at $-10°$ until required. Serum obtained from guinea pigs after sensitization to bovine insulin by this method is here termed insulin antiserum.

Hypoglycaemic agents. The actions of some synthetic hypoglycaemic agents upon glucose uptake of the isolated rat diaphragm were also studied; these included *n*-decamethylene-1:10-diguanide (Synthalin, L. Light and Co. Ltd., Bucks), *N'*-phenylethyl diguanide (phenethyl diguanide), *N*-(*n*-butyl)-*N'*-(*p*-aminophenyl)-sulphonyl urea (carbutamide) and *N*-(*n*-butyl)-*N'*-(*p*-methylphenyl)-sulphonyl urea (tolbutamide).

Glucose consumption of isolated rat hemi-diaphrams. The experimental procedure used in estimation of glucose uptake by rat hemi-diaphragms has been described in detail elsewhere (Wright, 1957). Wistar rats (120–140 g.), bred in Guy's Hospital Medical School from a strain supplied by the Chester Beatty Research Institute, were fasted for 24 hr., weighed and killed by decapitation. The diaphragms were removed, soaked in Krebs–Ringer bicarbonate buffer (Umbreit, Burris & Stauffer, 1945) at 0–8° for 15 min. The hemi-diaphragms were dissected, blotted on filter paper and then placed in Warburg flasks containing 2 ml. of incubation medium; in no experiment were the hemi-diaphragms from any one rat incubated in the same medium. After being gassed for 5 min. with $O_2 + CO_2$ (95:5), the flasks were incubated at 37° for 90 min. with continuous agitation (120 oscillations/min.; 4 cm. amplitude). The glucose concentration in each medium was determined before incubation and in each flask after incubation; a slight modification of King's method of glucose estimation was used (see Wright, 1957). On removal from the flasks the hemi-diaphragms were washed in water, blotted and weighed after drying in air at 120° for 2 hr. Glucose uptake of each individual hemi-diaphragm is expressed as μg. of glucose/10 mg. dry wt. of muscle/90 min. incubation.

Incubation media. The hemi-diaphragms were incubated in Krebs–Ringer bicarbonate buffer (Umbreit, Burris & Stauffer, 1945) containing glucose in a concentration of 280–300 mg./100 ml. This medium is termed 'saline buffer'. Insulin was incorporated by serial dilution of stock insulin solutions (usually 50 units/ml. in aq. 0·6% acetic

acid) with saline buffer immediately before each experiment. Guinea-pig or rabbit serum was added to the incubation medium after the addition of insulin or other hypoglycaemic agent and 1–1½ hr. before exposure to the tissue.

RESULTS

The glucose uptake of hemi-diaphragms incubated in saline buffer alone (basal uptake) was unaffected by the addition of serum from normal guinea pigs or of that from guinea pigs sensitized to bovine insulin (insulin antiserum) (Table 1). The uptake of muscle incubated in saline buffer containing insulin (1 m-unit/ml.) was also unaffected when normal guinea-pig serum was added, but was reduced when insulin antiserum was present (Table 2). The inhibitory effect of the insulin antiserum upon the action of bovine insulin appeared to depend upon the concentration of antiserum present. All five samples of insulin antiserum obtained at various times from three groups of guinea pigs (A, B and C) abolished the stimulant action of the standard concentration of insulin used (1 m-unit/ml.) provided that they were present in concentrations equal to or greater than 0·02 ml. of serum/ml. of incubation medium. It was also noted that serum drawn from guinea pigs 5 months after the last injection of antigen showed no inhibitory effect, though the inhibition returned after the next course of antigen injections. Specimens of antiserum stored at $-10°$ for 6 months showed no loss of inhibitory activity.

Insulin antiserum from one group of guinea pigs sensitized to bovine insulin also inhibited the stimulant effects produced by comparable concentrations of human, pig and sheep insulin. The stimulant effect of bovine insulin labelled with ^{131}I, like that of the recrystallized insulin from which it was produced, was also reduced (Table 3).

By contrast the stimulant effects of Synthalin and phenethyl diguanide (Table 4) were not reduced by the addition of insulin antiserum in a concentration known to be effective against bovine insulin. Carbutamide and tolbutamide are without stimulant action. The effects produced by these hypoglycaemic agents confirm the majority of findings previously reported (see Lundbaek, Nielson & Rafaelson, 1958).

A specimen of serum obtained from rabbits sensitized against bovine insulin and known to agglutinate insulin-sensitized red cells (titre 1/300) was kindly provided by Dr A. B. Stavitsky. When tested in the same way as the guinea-pig antiserum it produced no inhibitory effect even when present in high concentration (0·1 ml. of serum/ml. of incubation medium). It was thought possible that the action of this serum might depend upon the presence of complement which had been removed during heat-treatment of the serum. When fresh normal rabbit serum was also added, however, no inhibitory effect was observed (Table 5).

The insulin antiserum obtained from guinea pigs was fractionated by treatment with 19% sodium sulphate, and the globulin fraction, after dialysis, was found to possess the inhibitory activity. It was also shown that antiserum when added to bovine insulin before injection produced the protective effect described by Moloney & Coval (1955). Injection of bovine insulin (15 units) by the intracardiac route into sensitized guinea pigs produced typical symptoms of severe fatal anaphylaxis within 5 min., whereas only hypoglycaemic symptoms developed in normal guinea pigs in 2–3 hr.; none of the latter animals died.

Table 1. *Effect of serum from normal and insulin-sensitized guinea pigs upon glucose uptake of the isolated rat hemi-diaphragm*

For details of the saline buffer see Materials and Methods.

Guinea-pig serum			Uptake of glucose*	
Type	Batch	Concn. (ml./ml. of incubation medium)	Saline buffer alone (basal)	Saline buffer + serum
Normal	—	0·1	266±11·3 (7)	280±16·9 (7)
Anti-insulin	A_2	0·03	279±14·1 (6)	264±10·3 (5)
	B_2	0·03		264±15·2 (4)

* Uptake of glucose (μg. of glucose/10 mg. dry wt./90 min.±S.E.M.) of isolated hemi-diaphragms incubated in saline buffer alone or in buffer containing added guinea-pig serum; the number of hemi-diaphragms incubated is given in parenthesis.

Table 2. *Glucose uptake of rat hemi-diaphragms incubated in the presence of insulin and serum from normal and insulin-sensitized guinea pigs*

Mean glucose uptake of hemi-diaphragms incubated in each medium during each group of experiments is stated as μg. of glucose/10 mg. dry wt./90 min. ± S.E.M. The number incubated in each medium is shown in parentheses.

Serum no.	No. of expts.	Uptake of glucose									
		Saline buffer alone (basal uptake)	Saline buffer + insulin (1 m-unit/ml.)	Saline buffer + insulin (1 m-unit/ml.) + serum (ml. of serum/ml. of incubation medium)							
				0·1	0·05	0·04	0·03	0·02	0·01	0·002	0·001
Normal serum											
N	3	256 ± 16·3 (8)	502 ± 16·7 (8)	495 (3)	488 (4)	—	—	529 ± 30 (10)	—	—	—
Insulin antiserum											
A	5	264 ± 10·7 (13)	521 ± 15·6 (13)	252 (3)	—	311 ± 36 (5)	242 (2)	248 ± 23·6 (6)	248 (4)	—	—
A_1	3	212 ± 10·2 (14)	469 ± 21·1 (10)	—	—	162 (3)	—	267 ± 10·6 (9)	338 (3)	—	—
B_1	2	188 ± 15·5 (7)	393 ± 24·5 (6)	—	—	—	—	223 (3)	441 (3)	381 (4)	447 (3)
B_2	3	263 ± 12·0 (7)	458 ± 20·6 (10)	—	—	—	—	289 ± 17·5 (9)	—	—	—
C_1	2	249 ± 14·6 (8)	451 ± 18·7 (7)	—	283 (4)	—	—	211 (3)	—	—	—

Table 3. *Effect of serum from guinea pigs sensitized to bovine insulin upon the actions of insulin from various animal sources upon the isolated rat hemi-diaphragm*

In each experiment four hemi-diaphragms were incubated individually in each medium, the mean glucose uptake in each case being stated as μg. of glucose/10 mg. dry wt./90 min.; the S.D. in all cases was 30–50.

	Uptake of glucose			
			Saline buffer + insulin* + antiserum	
Source of insulin	Saline buffer alone (basal uptake)	Saline buffer + insulin*	0·05 ml. of antiserum/ml. of medium	0·02 ml. of antiserum/ml. of medium
Bovine	266	470	283	288
Bovine (^{131}I-labelled)	—	388	284	—
Human	285	493	254	307
Pig	284	485	314	320
Sheep	259	459	256	315

* Insulin concentration = approx. 1 m-unit/ml.

Table 4. *Glucose uptake of rat hemi-diaphragms incubated in the presence of synthetic hypoglycaemic agents and insulin antiserum*

Hemi-diaphragms were incubated in saline buffer in the presence of the hypoglycaemic agent and, with active agents, insulin antiserum (concentration 0·05 ml. of serum/ml. of incubation medium). Uptake of glucose of hemi-diaphragms incubated in each medium (number given in parentheses) is given as μg. of glucose/10 mg. dry wt./90 min. ± S.E.M.

		Uptake of glucose		
Hypoglycaemic agent	Concn. of agent (mg./100 ml.)	Saline buffer alone	Saline buffer + agent	Saline buffer + agent + antiserum
Carbutamide	50	263 ± 6 (8)	244 ± 19 (8)	—
Tolbutamide	50	289 ± 12 (8)	274 ± 23 (8)	—
Synthalin	5	231 ± 16·8 (5)	339 ± 21·4 (5)	444 ± 8·0 (6)
Phenethyl diguanide	50	261 ± 20·1 (5)	374 ± 38 (5)	441 ± 21 (6)

Table 5. *Effect of serum from insulin-sensitized rabbits upon the action of bovine insulin on the isolated rat hemi-diaphragm*

Hemi-diaphragms were incubated individually in saline buffer with or without addition of insulin (1 m-unit/ml.), normal rabbit serum (0·05 ml. of serum/ml. of medium) or serum from insulin-sensitized rabbits. The mean glucose uptake of hemi-diaphragms incubated in each medium (number shown in parentheses) is stated as μg. of glucose/10 mg. dry wt./90 min.; the S.D. in each case was 30–50. When added alone, insulin antiserum was added in concentrations of 0·1, 0·05 and 0·02 ml. of serum/ml. of medium, but when added with normal serum its concentration was 0·05 ml. of serum/ml. of medium.

Uptake of glucose						
				Saline buffer + insulin + antiserum		
Saline buffer alone	Saline buffer + insulin	Saline buffer + insulin + normal serum	Saline buffer + insulin + normal serum + insulin antiserum	0·1 ml. of antiserum/ml. of medium	0·05 ml. of antiserum/ml. of medium	0·02 ml. of antiserum/ml. of medium
250 (4)	—	476 (4)	476 (4)	—	456 (4)	—
199 (2)	431 (3)	—	—	455 (3)	—	440 (4)

DISCUSSION

These results demonstrate that serum from guinea pigs sensitized to bovine insulin will inhibit an effect produced by insulin *in vitro*. This inhibition does not appear to be species-specific, since serum from guinea pigs sensitized to insulin extracted from the pancreas of one animal (ox) abolishes the stimulant effect of insulin extracted from the pancreases of others (sheep, pigs and human beings). It is specific for insulin only in so far as it does not abolish the stimulant effects of the synthetic hypoglycaemic agents tested.

Moloney & Coval (1955) showed that such insulin antiserum neutralizes the hypoglycaemic effect of insulin *in vivo*, and that this neutralizing effect was not species-specific. They concluded that it was due to the presence in the guinea-pig serum of insulin antibodies. Since then P. J. Moloney (personal communication, 1958) has demonstrated that such serum will precipitate insulin *in vitro* and that the precipitate has the properties of an antigen–antibody complex. Arquilla & Stavitsky (1956*a*) showed that serum obtained from rabbits after prolonged treatment with intravenous alum-precipitated insulin will agglutinate red cells conjugated with insulin; this effect is abolished if the serum is first incubated in the presence of insulin. They concluded (1956*b*) that this effect is due to the presence in the serum of antibodies which are specific but not species-specific for insulin. The neutralizing effect of serum from insulin-treated animals and human beings has been demonstrated in many laboratories (*vide supra*) and it has been shown that the active component of the serum is a γ-globulin (Sehon *et al.* 1955). However, serum obtained from insulin-sensitized rabbits and known to agglutinate insulin-conjugated red cells did not inhibit the action of bovine insulin upon the isolated rat diaphragm either in the presence or the absence of normal rabbit serum (Table 5). This failure could be due to the low titre of the antiserum (1/300), to loss of activity during transit by post or to the long interval between the exposure of the serum to insulin and subsequent introduction of the isolated muscle (60–90 min.). On the other hand, rabbits are extremely resistant to the sensitizing effects of insulin (see Moloney & Goldsmith, 1957) and the protective effect of serum obtained from insulin-sensitized rabbits is much less pronounced than that of serum from sensitized guinea pigs. It is possible therefore that the antibodies which inhibit the physiological effects of insulin upon the intact animal or isolated muscle are not identical with those which react with insulin-conjugated red cells.

With the present technique it is possible to abolish the stimulant effects of small amounts of insulin which are insufficient to produce significant effects on injection into mice. It can therefore be used to aid in the identification of small amounts of biologically active material in tissues or tissue extracts. Thus fractions of bovine-plasma proteins (Taylor, 1959; Wright, 1959) and extracts of tissue from a woman murdered by injections of insulin (Birkinshaw *et al.* 1958) were found to stimulate glucose consumption of the isolated rat diaphragm and this stimulant effect was abolished after treatment with insulin antiserum. In each case the active component had other properties which were identical with those of the pure hormone; it was destroyed by pepsin and cysteine and the biological response was qualitatively the same.

Moloney & Coval (1955) pointed out that such antiserum does not always neutralize the effect of insulin *in vivo*. Thus the animal undergoing sensitization is normoglycaemic; the antibodies produced do not neutralize the insulin secreted by the pancreas of the sensitized animal. A sheep which was sensitized to pig insulin was resistant to the hypoglycaemic effect of crystalline pig and sheep insulin. These results suggest that there is an immunological difference between the endogenous insulin secreted by the animal's pancreas and the crystalline, or altered, insulin extracted from it. In addition insulin extracted from the guinea-pig pancreas is not neutralized by serum from guinea pigs sensitized to bovine or sheep insulin (Moloney & Goldsmith, 1957). Thus besides some immunological differences between endogenous and altered insulins from the same animal, there are also differences, well recognized chemically (Brown, Sanger & Kitai, 1955; Goldsmith & Moloney, 1957), between insulins extracted from the pancreases of different animals. It is likely therefore that insulin preparations will be obtained whose stimulant effects upon the rat diaphragm will not be abolished by insulin antiserum prepared by the methods described above. It seems reasonable to conclude, however, that if the stimulant effect of a biologically active material upon the isolated rat diaphragm is abolished by insulin antiserum, that material must contain insulin.

SUMMARY

1. Serum obtained from guinea pigs sensitized to bovine insulin inhibits the increase normally produced by bovine insulin upon the glucose uptake of isolated rat diaphragm; serum from normal guinea pigs has no such action.

2. This inhibition is not species-specific in that serum from guinea pigs sensitized to insulin from one animal source (cattle) will inhibit the effects produced by insulin obtained from others (pigs, sheep and human beings).

3. The stimulant effects of the synthetic hypoglycaemic agents Synthalin and phenethyl diguanide are not affected by insulin antiserum.

4. The significance of these findings is discussed and it is suggested that such insulin antiserum could be used to aid in the identification of insulin in extracts from biological materials.

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Studies in Detoxication

78. GLUCURONIDE AND ETHEREAL SULPHATE FORMATION IN HENS*

By B. C. BALDWIN, D. ROBINSON and R. T. WILLIAMS
Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

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The fate of foreign organic compounds in the domestic fowl has not been extensively investigated. Most of the earlier studies were concerned with the synthesis of ornithuric acid (N^2N^5-dibenzoylornithine, first discovered by Jaffe in 1877) because the fowl appeared to differ from most other species, since it conjugated aromatic acids with ornithine rather than with glycine. Preliminary work in this Laboratory with [^{14}C]benzoic acid suggests that ornithuric acid formation may account for nearly half of moderate doses of benzoic acid. References to other reactions of foreign compounds in hens are few, e.g. acetylation (Crowdle & Sherwin, 1923; Shaffer & Beiter, 1950), methylation (Hoshiai, 1909; Takahashi, 1927; Tamura, 1924), oxidation and reduction (Crowdle & Sherwin, 1923). Sperber (1947) found that phenols injected into hens caused an increase in the excretion of ethereal sulphates and of glucuronic acid derivatives as measured by the naphtharesorcinol reaction, but injected benzoic acid, *p*-aminobenzoic acid and chloral did not increase glucuronic acid excretion. This suggested that hens could form the ether glucuronides of phenols, but not the ester glucuronides of aromatic acids. Wu Chang & Johnson (1957), however, claim to have detected by paper chromatography nicotinoyl glucuronide, an ester glucuronide, in the urine of chicks giving nicotinamide or nicotinic acid. In the present work we have shown, by isolation of the

* Part 77: McIsaac, Parke & Williams (1958).

conjugates, that the domestic fowl can synthesize appreciable amounts of both ether and ester glucuronides and of ethereal sulphates.

MATERIALS AND METHODS

Diet and animals. The hens used were Rhode Island Red crossed with Light Sussex, weighing 2·5–3·9 kg. They were maintained on 150 g. of mash/day (Superbra battery, intensive mash, Poultry Association of Great Britain). Compounds were administered to the hens by crop tube. Usually the compounds were fed at a dose level of 2 m-mole/kg., dissolved or suspended in 25 ml. of water to which a knife-point of bile salt had been added.

Collection of excreta and analytical methods. In studies with hens one of the main difficulties is the collection of urine and of faeces separately. We found that the determination of glucuronic acid and ethereal sulphate in mixed urine and faeces was completely unsatisfactory. Various techniques have been described for the separation of urine from faeces. Surgical operations for separating the rectum from the cloaca and for exteriorizing the ureters have been criticized as leaving the hen in an abnormal condition. Cannulation of the ureters has been used, but this is difficult since the ureters are so small and difficult to locate. Hart & Essex (1942) used a special cannula fitted with a glass ball to block the rectum, but this can be used only for periods of less than 2 hr., which were insufficient for our experiments. We found, however, that if food was withheld

Table 1. *Glucuronide excretion by hens given borneol*

On days 1, 3, 6, 7, 8, 10 and 12 food was given to the hens. DL-Borneol (304 mg./kg.) was given on day 4.

	Hen 15 (2·7 kg.)		Hen 16 (2·7 kg.)		Hen 17 (2·4 kg.)	
Day	Glucuronic acid output (mg.)	Urine vol. (ml.)	Glucuronic acid output (mg.)	Urine vol. (ml.)	Glucuronic acid output (mg.)	Urine vol. (ml.)
2	3·6	22	2·7	13	6·2	31
4	205·0	100	198·0	40	174·0	68
5	58·5	90	76·7	26	5·1	17
9	6·8	109	11·2	20	6·9	15
11	5·8	92	1·2	19	14·0	35
13	4·0	80	3·6	17	3·1	8
Average normal value	5·0	—	4·7	—	7·5	—
Excess after borneol	253·5	—	265·3	—	166·5	—
Percentage of dose conjugated with glucuronic acid	25	—	26	—	18	—

Table 2. *R_F values of some glucuronides and ethereal sulphates*

R_F values are for descending chromatography on Whatman no. 4 paper. Solvent mixtures: *A*, in butanol–acetic acid–water (4:1:2, by vol.) run for 6 hr.; *B*, butanol–benzene–pyridine–water (5:1:3:3, by vol.) run for 4 hr.; *C*, propanol–aq. NH_3 soln. (sp.gr. 0·88) (7:3, by vol.) run for 5 hr.; *D*, butanol–acetic acid–water (4:1:5, by vol.) run for 6 hr. In the times quoted the solvent front moved about 30 cm. from the origin.

Sprays used: *a*, aq. 1% naphtharesorcinol followed by heating at 110° in an atmosphere of HCl gas, which gave dark-blue spots with glucuronic acid and glucuronides; *b*, snail digestive juice (diluted with 10 vol. of water; see Jarrige & Henry, 1952) followed by incubation at 37° for at least 1 hr. between glass plates, and then spraying with aq. 0·1% 2:6-dichloroquinone chloroimide followed by saturated $NaHCO_3$, which gave blue spots with certain phenolic glucuronides and sulphates. *m*-Aminophenylsulphate was detected as a red spot, which turned brown on keeping, by spraying successively with 0·1% $NaNO_2$ in 0·2N-HCl, 1% ammonium sulphamate and 0·5% α-naphthylamine in diluted ethanol, allowing the paper to dry between each spray. Illumination of the paper with u.v. light from a low-pressure mercury arc lamp (Hanovia Chromatolite) revealed *o*-methoxybenzoic acid and its glucuronide as purple fluorescent spots, and glucurone and glucuronic acid quenched the background fluorescence of the paper.

	R_F values			
Solvent mixture	*A*	*B*	*C*	*D*
Compound				
Glucuronic acid*	0·28	—	0·07	—
Glucurone	0·49	0·64	{0·07 {0·23	—
Bornylglucuronide	0·91	0·59	0·76	—
o-Methoxybenzoic acid	—	0·65	—	0·94
o-Methoxybenzoylglucuronide	—	0·26	—	0·58
Phenylglucuronide	—	0·22	0·45	—
Phenylsulphate†	—	0·60	0·84	—
m-Aminophenylsulphate†	0·45	0·35–0·41	0·65	—

* Applied to the paper as the sodium salt.

† Applied as the potassium salt.

from the hen for 24 hr., a greenish fluid containing little or no solid faeces was excreted. This fluid, which we assumed to be mainly urine, could be used satisfactorily for the determination of glucuronic acid and ethereal sulphate.

The technique eventually adopted was to give the hen a double ration of food (300 g.) on one day and none on the second day. Most of the food was eaten on the first day and the excreta for this day were discarded; the excreta for the second day contained little or no solid matter and were collected and analysed. When foreign compounds were administered, food was withheld for 48 hr. and quantitative estimations were carried out on the urine excreted on these 2 days. Glucuronic acid was determined according to Paul (1951; see Mead, Smith & Williams, 1958) and under the above conditions the average normal excretion of glucuronic acid was 7·5 mg./day (range 1·2–18·5 mg.) (28 determinations on six hens). Typical quantitative results are given in Table 1. Determination of ethereal sulphate was carried out by the method of Folin (1905–06). The turbidimetric method of Sperber (1948) gave completely unsatisfactory results. The average ethereal sulphate output was 3·8 mg. of SO_3/day (range 0·4–8·5 mg.) (22 determinations on six hens).

Chromatography. R_F values for the compounds used are shown in Table 2.

ISOLATION AND DETECTION OF GLUCURONIDES AND ETHEREAL SULPHATES

Glucuronide of chloroxylenol

From rabbits. Two rabbits were given, by stomach tube, 2 g. each of chloroxylenol (4-chloro-3:5-dimethylphenol) and their urine was collected for 24 hr. The glucuronide was separated as the basic lead salt (Kamil, Smith & Williams, 1951). After removal of the lead, the aqueous solution of the glucuronide was concentrated *in vacuo* at 40–50° to a jelly which did not crystallize. It was therefore dried *in vacuo* and acetylated with acetic anhydride containing one drop of 60% perchloric acid. On diluting with water, *4-chloro-3:5-dimethylphenyl triacetylglucosiduronic acid* (0·3 g.) separated as white plates, m.p. 201–202° after recrystallization from methanol; $[\alpha]_D^{21} - 22 \pm 1°$ in ethanol (*c*, 1) (Found: C, 52·15; H, 5·35. $C_{20}H_{23}O_{10}Cl$ requires C, 52·35; H, 5·05%). In another experiment the glucuronide fraction was prepared from the urine of a rabbit which had been given 1·5 g. of chloroxylenol. The dried glucuronide fraction prepared as above was first methylated with diazomethane and then acetylated with pyridine and acetic anhydride (see Kamil *et al.* 1951). On diluting the acetylation mixture with water, *methyl (4-chloro-3:5-dimethylphenyl tri-O-acetyl-D-glucosid)uronate* (0·5 g.) separated as colourless needles, m.p. 176° after recrystallization from methanol, and $[\alpha]_D^{21} - 30 \pm 1°$ in $CHCl_3$ (*c*, 1) (Found: C, 53·7; H, 5·6. $C_{21}H_{25}O_{10}Cl$ requires C, 53·3; H, 5·3%). The ester (100 mg.) in 4N-H_2SO_4 (20 ml.) was boiled under reflux for 2 hr. and then the mixture was steam-distilled. From the distillate, chloroxylenol (20 mg.) was recovered, m.p. and mixed m.p. 114°.

An attempt was also made to prepare free chloroxylenol glucuronide. The 24 hr. urine of two rabbits, each dosed with 2 g. of chloroxylenol, was brought to pH 3–4 by adding 2N-H_2SO_4, and then continuously extracted with ether for 24 hr. Crystals (0·1 g.) separated in the ether and appeared to be chloroxylenol glucuronide (m.p. 202–206° and $[\alpha]_D^{20} - 56°$ in water) but recrystallization from a variety of solvents gave gels. On acetylation, however, the triacetyl acid (m.p. 202° and $[\alpha]_D^{20} - 22°$ in ethanol) was obtained.

From hens. Two hens which had been starved overnight were each given 2 g. of chloroxylenol in water by crop tube. The mixed urine and faeces were collected for the next 2 days. The first day's excreta were centrifuged. The supernatant fluid was brought to pH 2–3 with 2N-H_2SO_4 and continously extracted with ether for 24 hr. The extract was dried over anhydrous Na_2SO_4 and then treated with an excess of diazomethane in ether. The ether was evaporated and the dry residue treated with acetic anhydride (4 ml.) and 1 drop of 60% perchloric acid. When the reaction had subsided, the mixture was diluted with water and the crystals (0·38 g.) which separated were collected. After recrystallization from ethanol, the white needles obtained were identified as methyl (4-chloro-3:5-dimethylphenyl tri-*O*-acetyl-D-glucosid)uronate, m.p. and mixed m.p. 175–176° and $[\alpha]_D^{20} - 31·6°$ in $CHCl_3$ (*c*, 0·9).

m-*Cresyl glucuronide*

From rabbits. *m*-Cresol (2 g.) in water was administered to a rabbit by stomach tube. The 24 hr. urine was collected and the glucuronide fraction separated by the lead acetate method (Kamil *et al.* 1951). The glucuronide gum was methylated and acetylated to give 3·5 g. of crude methyl (*m*-cresyl tri-*O*-acetylglucosid)uronate. The compound recrystallized from ethanol and light petroleum (b.p. 100–120°) had m.p. 109° and $[\alpha]_D^{20} - 35°$ in $CHCl_3$ (*c*, 1) (Found: C, 56·7; H, 5·8; OMe, 7·6. Calc. for $C_{20}H_{24}O_{10}$: C, 56·6; H, 5·7; OMe, 7·35%). Kamil *et al.* (1951) give m.p. 93–94° and $[\alpha]_D - 25·6°$, but their compound appears to have been contaminated with a second glucuronide which occurs to the extent of about 10% of the glucuronide fraction and is being investigated by one of us (D.R.).

From hens. *m*-Cresol (1 g.) in aqueous suspension was administered orally to a hen (3·5 kg.). Food was withheld overnight and in the morning 100 ml. of thick green urine was collected. The urine was diluted with an equal volume of acetone and the precipitate which formed was discarded. The acetone was evaporated *in vacuo* and the residual solution subjected to the lead acetate procedure (Kamil *et al.* 1951). The glucuronide was obtained as a neutral gum which was dissolved in water (20 ml.) and then treated with Zeo-Karb 225 (acid form; 0·2 g.). The resin was filtered off and the filtrate evaporated *in vacuo* to an acid gum (0·2 g.). The gum was methylated and acetylated to yield 0·28 g. of methyl (*m*-cresyl tri-*O*-acetylglucosid)uronate, m.p. and mixed m.p. 109°, and $[\alpha]_D^{20} - 31·5°$ in $CHCl_3$ (*c*, 2) after several recrystallizations from ethanol and light petroleum (b.p. 100–120°).

Benzoyl glucuronide

From dogs. Methyl (benzoyl glucosid)uronate had been prepared (cf. Quick, 1926) from a sample of benzoyl glucosiduronic acid obtained by Pryde & Williams (1933) by feeding dogs with benzoic acid. The ester, which reduced Benedict's reagent on heating, had m.p. 186° and $[\alpha]_D^{21} - 16·1°$ in methanol (Found: C, 53·5; H, 5·1; OMe, 9·3. Calc. for $C_{14}H_{16}O_8$; C, 53·8; H, 5·2; OMe, 9·9%).

Table 3. *Absorption spectra in ethanol of benzoic acid and its conjugates*

	$\lambda_{max.}$		Corresponding $\epsilon_{max.}$	
Benzoic acid	229	273	12 000	860
Benzoyl glucosiduronic acid (from hen urine)	230·5	275	14 300	1270
Methyl (benzoyl glucosid)uronate (from dog urine)	230	275	13 900	1070
Methyl (benzoyl glucosid)uronate (from hen urine)	230	275	13 300	1110
Hippuric acid	226	~269	10 750	About 700

From hens. Two hens, deprived of food overnight, were each given 1 g. of benzoic acid in water by crop tube. The urine was collected for 18 hr., brought to pH 2–3 with 2N-H_2SO_4 and continuously extracted with ether for 16 hr. The extract was dried over anhydrous Na_2SO_4 and evaporated to a thick syrup, which was then taken up in $CHCl_3$ (150 ml.). This solution was kept overnight at 0° and a white amorphous solid (150 mg.) separated. From its solution in the least volume of ethanol benzoyl glucosiduronic acid was precipitated by light petroleum (b.p. 40–60°) as a white powder, m.p. 180° and $[\alpha]_D^{20}$ – 25·6° in water (*c*, 0·7), which readily reduced Benedict's reagent and gave an intense naphtharesorcinol reaction. Goebel (1938) gives m.p. 184–185° and $[\alpha]_D$ – 26·8° in water. The absorption spectrum of the acid was similar to that of benzoic acid (see Table 3).

The acid (30 mg.) was converted into its methyl ester with ethereal diazomethane at −15°. This ester reduced Benedict's reagent and, after recrystallization from water, it was obtained as white needles, m.p. 191–193° and mixed m.p. 190–191°, $[\alpha]_D^{21}$ – 17·5° in methanol (*c*, 0·4). Its absorption spectrum was identical with that of the ester obtained from dog urine (see Table 3). A small amount (15 mg.) was acetylated with acetic anhydride (1 drop) and pyridine (1 drop) to yield methyl (benzoyl tri-*O*-acetylglucosid)uronate, m.p. 143–144°. Goebel (1938) gives m.p. 145°.

o-*Methoxybenzoyl glucuronide*

From rabbits. *o*-Methoxybenzoic acid (2 g.) was administered to each of four rabbits and the urine was collected for the subsequent 24 hr. This urine reduced Benedict's reagent on warming and gave an intense naphtharesorcinol reaction. The glucuronide was separated by the lead acetate procedure of Kamil *et al.* (1951). It was obtained as white needles (5 g.) and was purified by repeated recrystallization from ethanol. The compound separated from this solvent as white needles containing one molecule of ethanol of crystallization, m.p. 121–122° and $[\alpha]_D^{20}$ – 34·5±2° in water (*c*, 1). The *o-methoxybenzoyl glucosiduronic acid ethanolate* reduced Benedict's reagent on warming (Found: C, 51·2; H, 5·6; (OMe + OEt)/2, 21·5. $C_{14}H_{16}O_9,C_2H_5OH$ requires C, 51·35; H, 5·9; 1 OMe + 1 OEt, 20·3%). *Methyl* (*o-methoxybenzoyl tri-O-acetyl-D-glucosid*)*uronate* was prepared by methylation and acetylation of the preceding ethanolate and formed white needles, m.p. 138° and $[\alpha]_D^{20}$ – 7±2° in $CHCl_3$ (*c*, 1·5) (Found: C, 54·15; H, 5·5; OMe, 13·3; $C_{21}H_{24}O_{12}$ requires C, 53·8; H, 5·2; OMe, 13·2%).

From hens. Three hens were given about 0·8 g. each of *o*-methoxybenzoic acid in water by crop tube. Paper chromatography of the urine in two solvents showed *o*-methoxybenzoyl glucuronide to be present. Attempts to isolate the glucuronide or a derivative were not successful.

m-*Aminophenylsulphate*

Preparation of 5-aminoacridine m-*aminophenylsulphate.* This salt was prepared by adding an excess of hot saturated aqueous solution of 5-aminoacridine hydrochloride to a saturated aqueous solution of potassium *m*-aminophenylsulphate (prepared according to Smith, 1955). The *salt* immediately separated from the solution as yellow needles, m.p. 240° after several recrystallizations from hot water containing 10–20% of ethanol (Found: SO_4, 24·8; $C_6H_6NSO_4^-$, 49·3; $C_{12}H_{10}N_2$, 50·8. $C_{19}H_{17}N_3SO_4$ requires SO_4, 25·05; $C_6H_6NSO_4^-$, 49·1; $C_{13}H_{10}N_2$, 50·6%). Sulphate was determined turbidimetrically (Sperber, 1948), *m*-aminophenylsulphate colorimetrically (Smith, 1955) and 5-aminoacridine fluorimetrically.

m-*Aminophenylsulphate from hen excreta.* Two hens (each weighing 2 kg.) were given 0·5 g. of *m*-aminophenol each. The phenol was suspended in water containing a little bile salt and administered by crop tube in the morning, the birds having last been given food the previous evening. The hens were given water *ad lib.* but no food for 24 hr. The excreta produced in this time were mainly liquid; they were pooled and centrifuged. The supernatant liquid was saturated with K_2CO_3 and then extracted with acetone (4 × 250 ml.) which had been kept in contact with saturated K_2CO_3 solution. The extract was filtered and evaporated under reduced pressure to 20 ml. The residue was transferred to a beaker and treated with 5–10 ml. of hot saturated aqueous 5-aminoacridine hydrochloride solution until the yellow precipitate flocculated. After being kept at 0° overnight, the yellow crystals were collected and recrystallized (charcoal) several times from hot water containing 10–20% of ethanol. The crystals were identified as 5-aminoacridine *m*-aminophenylsulphate, m.p. and mixed m.p. 239–240° (yield 0·61 g. or 17·4% of the dose) (Found: SO_4, 24·3; $C_6H_6NSO_4^-$, 51·0; $C_{13}H_{10}N_2$, 50·5%). The potassium *m*-aminophenylsulphate prepared from this salt showed R_F values in three solvents identical with those of the synthetic sample (see Table 2).

Phenylglucuronide and phenylsulphate were detected by paper chromatography in the urine of phenol-fed hens. The formation of bornylglucuronide after administration of (±)borneol was also shown to occur.

Table 4. *Glucuronic acid conjugation in hens*

Compounds were administered orally (dose 2 m-mole/kg.). Figures quoted are the average for three animals, the range being in parentheses.

	Percentage of dose excreted conjugated			
	By hens		By rabbits	
Compound fed	As glucuronide	As ethereal sulphate	As glucuronide	As ethereal sulphate
Benzoic acid	36 (28–47)	—	15*	—
(±)Borneol	23 (18–26)	2 (1–3)†	26 (24–29)	—
Chloroxylenol	27 (23–31)	—	53 (43–71)	21 (17–27)
o-Methoxybenzoic acid	26 (21–34)	1 (0·5–1·5)†	67 (62–75)	—

* Quoted from Bray, Humphris, Thorpe, White & Wood (1955). † Probably not significant.

RESULTS AND DISCUSSION

Quantitative results on the glucuronic acid output of hens dosed with two aromatic acids, a phenol and a terpene secondary alcohol, are given in Table 4, which shows that glucuronic acid conjugation is a mechanism of importance in the hen since it can account for nearly one-third of the dose of these compounds. Furthermore, hens can form glucuronides of both the ether and ester type. Chloroxylenol and *o*-methoxybenzoic acid are more extensively conjugated with glucuronic acid in rabbits than in hens, but with benzoic acid the reverse seems to be true. In the rabbit, glycine conjugation is the major mechanism by which benzoic acid is metabolized and it can account for 80% or more of the benzoic acid fed (Bray, Humphris, Thorpe, White & Wood, 1955). Glycine conjugation of benzoic acid does not occur in hens and is replaced by ornithine conjugation, which can account for up to 50% of a dose of 2 m-mole of benzoic acid/kg. (B. C. Baldwin, D. Robinson & R. T. Williams, unpublished work). Borneol seems to be equally well conjugated with glucuronic acid in the hen and the rabbit.

Sulphate conjugation of phenols also occurs in hens in which *m*-aminophenol is conjugated with sulphate to at least 17–18% of the dose (0·25 g./kg.) based on isolation; the sulphate conjugation of *m*-aminophenol in rabbits is 15% of the dose (1 g./rabbit) (Bray, Clowes & Thorpe, 1952).

SUMMARY

1. Phenol, *m*-cresol, 4-chloro-3:5-dimethylphenol, borneol, benzoic acid and *o*-methoxybenzoic acid when fed to hens are excreted as conjugated glucuronic acids. Glucuronic acid conjugation is a mechanism of importance in hens and can account for about a third of moderate doses of the last four of these substances.

2. The glucuronides of *m*-cresol, chloroxylenol and benzoic acid were isolated from chicken urine as crystalline derivatives and were compared with the same derivatives isolated from rabbit or dog urine.

3. Phenols are conjugated with sulphate in hens. The ethereal sulphate of *m*-aminophenol was isolated from hen urine as the 5-aminoacridine salt.

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Nitrogenous Compounds and Nitrogen Metabolism in the Liliaceae

6. CHANGES IN NITROGENOUS COMPOSITION DURING THE GROWTH OF *CONVALLARIA* AND *POLYGONATUM**

By L. FOWDEN
Department of Botany, University College, Gower Street, London, W.C. 1

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Azetidine-2-carboxylic acid has been recognized as an important constituent of the 70% ethanol-soluble-nitrogen fraction of many liliaceous plants (Fowden, 1956; Fowden & Steward, 1957). *Convallaria majalis* (lily of the valley) and *Polygonatum multiflorum* (a Solomon's seal) are two common representatives of these plants in Britain, and part 5 of this series (Fowden & Bryant, 1959) described experiments in which various ^{14}C-labelled substances were supplied to tissues of these two plants to obtain information about the manner of biogenesis of azetidine-2-carboxylic acid. In this paper variations in the major nitrogenous fractions present in different organs of the plants during growth have been investigated to ascertain in another way where azetidine-2-carboxylic acid is synthesized and at what stage of the plant's development synthesis occurred. The importance of the imino acid in the general nitrogen economy of the plants is discussed.

EXPERIMENTAL

All plants were grown outdoors on the same location. Samples were collected at four stages of growth (S1, S2, S3 and S4). The first collection (S1) was made in February when the shoots remained as small underground buds. Collection S2 was made in mid-April. The *Convallaria* shoots were then 6–8 cm. high, the leaves being still rolled. The *Polygonatum* shoots were 12–15 cm. high, but the first leaves were not yet fully expanded. Collection S3 was made in late May when both plants were nearly mature. The *Convallaria* leaves were unfolded and had attained nearly maximum size, whereas the *Polygonatum* shoots had grown to their full height (60–80 cm.) and gained their maximum number of leaves. Both plants were in flower at this stage. The final collection S4 was taken in August when the shoots of both plants were becoming senescent and the leaves were browning. At each stage the plants were separated into root, rhizome and shoot components before analysis and their dry weight/fresh weight ratios determined. Since both plants are perennials the percentage increases in the shoot weights occurring during one season's growth were far higher than the corresponding increases for rhizome or roots.

Fractionation of the nitrogen constituents. Washed plant tissues were dried at 95° for 1 hr. and then at 65° to constant weight. The dried materials were ground to homogeneous powders upon which total nitrogen determinations were made. Other batches of plant material were macerated in an Ato-Mix Blender with 75% (v/v) ethanol (10 ml./g. fresh wt. of material). Extraction at laboratory temperature was continued for 24 hr. with shaking and, after separation of the extract and residue by centrifuging, the residue was twice re-extracted. The extracts were evaporated to dryness at temperatures below 50° and the residues were redissolved in water. Any material remaining insoluble at this stage was removed by centrifuging and discarded. Measured portions of these solutions were used for determinations of total soluble nitrogen and of individual free amino and imino acids.

Nitrogen determinations. Nitrogen was assayed by the micro-Kjeldahl method of Chibnall, Rees & Williams (1943).

Amino acid determinations. Individual acids were determined after their separation on two-dimensional paper chromatograms. The methods used have been described previously (Fowden & Bryant, 1959; Fowden & Webb, 1958; Fowden, 1954). A preliminary separation of the cationic fraction (amino acids) from each extract was made (Fowden & Webb, 1958) before paper chromatograms were prepared. The volumes of the cationic fractions were adjusted so that 1 ml. was equivalent to 2 g. fresh wt. of plant material, and 50 μl. of each was applied to the chromatograms (Whatman no. 3MM chromatographic-grade filter paper). Phenol–aq. NH_3 soln. (Dent, 1947) was used as the first developing solvent, and was followed by butan-1-ol–acetic acid–water (Partridge, 1948). The final chromatogram size was 27 cm. × 27 cm. Location of the amino acid spots and the quantitative determination of each followed exactly the procedure described by Fowden (1954). Only the principal free amino acids were assayed; these included glutamic acid, glutamine, asparagine, alanine, serine, arginine and azetidine-2-carboxylic acid. The reported values are means of triplicate determinations and are subject to errors up to ±10% of the recorded values.

Although azetidine-2-carboxylic acid gives a brown spot after treatment of qualitative paper chromatograms with ninhydrin, the imino acid reacts to give a normal bluish purple colour (absorption maximum λ 570 mμ) under the controlled conditions used in quantitative colour development. Colour production from azetidine-2-carboxylic acid was, however, only about 25% of that obtained when an equivalent amount of an α-amino acid reacted with ninhydrin.

* Part 5: Fowden & Bryant (1959).

RESULTS

Both plants have perennial rhizomes and roots but produce completely new shoots each year. During one season's growth the percentage changes in the dry weight of rhizome and root components are very small compared with those of the shoots. For instance in *Convallaria* the dry weights of the shoot at S2, S3 and S4 were approximately 2·6, 15 and 40 times the corresponding weight at S1.

The total- and soluble-nitrogen contents of the plants' organs at the different stages of development are presented in Tables 1 and 2 respectively. The dry-weight/fresh-weight ratios of the tissues are given in parentheses in Table 1. Active growth of the plants began between S1 and S2 and was presumably associated with a marked uptake of nutrients which led to increased total nitrogen contents throughout the plant. This increase was particularly pronounced for *Polygonatum* shoots, which attained a very high 'crude protein' content (about 48% of the dry weight) at S2. As further growth of the shoot occurred and the leaves reached maturity their total nitrogen content rapidly decreased. Smaller decreases were observed for the rhizome and root tissues between S2 and S4.

In *Convallaria* the variations in the soluble-nitrogen contents of the tissues followed the same pattern as those of total nitrogen. Maximum values were generally reached at S2. The increases observed for the shoot and root between S1 and S2 and the subsequent decreases to S4 tended to be proportionately greater than those observed for the total nitrogen content of the same organ. Soluble nitrogen at S2 therefore represented a larger percentage of the total nitrogen of these organs than it did at any other stage of growth (see parentheses, Table 2). The ratio soluble nitrogen/total nitrogen was generally higher in the rhizome than in the other organs. The most striking feature of the soluble-nitrogen data for *Polygonatum* was also the remarkably high levels found in the rhizome, particularly when expressed as percentages of the total nitrogen (see Table 2). These latter percentages were appreciably higher than the corresponding ones determined for *Convallaria* rhizome and somewhat higher than those recorded by Steward & Thompson (1954) for other typical storage organs, e.g. potato tuber, approximately 70%, and carrot root, approximately 60%.

The concentrations of individual free amino acids in the plant tissues are presented in Table 3. Well-marked trends in the concentrations were

Table 1. *Total nitrogen contents of* Convallaria *and* Polygonatum *tissues at different stages of growth*

Results are expressed as mg. of total nitrogen/g. dry wt. of tissue. Figures in parentheses are dry wt./fresh wt. ratios.

Sampling time	Root	Rhizome	Shoot
	Convallaria		
S1; early February	24·5 (0·361)	15·2 (0·321)	47·6 (0·237)
S2; mid-April	30·7 (0·226)	20·7 (0·268)	49·4 (0·134)
S3; late May	21·2 (0·247)	16·3 (0·233)	34·6 (0·226)
S4; August	12·9 (0·262)	12·3 (0·435)	17·6 (0·346)
	Polygonatum		
S1; early February	21·6 (0·177)	16·7 (0·190)	51·0 (0·180)
S2; mid-April	26·4 (0·159)	23·6 (0·161)	76·5 (0·153)
S3; late May	19·4 (0·152)	14·2 (0·156)	42·1 (0·179)
S4; August	15·5 (0·169)	13·7 (0·183)	27·5 (0·233)

Table 2. *The ethanol* 75% (*v*/*v*) *soluble-nitrogen contents of* Convallaria *and* Polygonatum *tissues at different stages of growth*

Results are expressed as mg. of soluble nitrogen/g. dry wt. of tissue. Figures in parentheses show soluble-nitrogen contents as percentages of the total nitrogen content of tissue.

Sample	Root	Rhizome	Shoot
	Convallaria		
S1	4·63 (18·9)	5·98 (39·3)	5·32 (11·2)
S2	9·91 (32·3)	6·60 (31·9)	12·6 (25·7)
S3	6·38 (30·1)	7·25 (44·5)	5·40 (15·6)
S4	2·79 (21·6)	3·26 (26·5)	1·59 (9·0)
	Polygonatum		
S1	5·63 (26·1)	13·2 (79·0)	13·3 (26·1)
S2	3·90 (14·8)	19·5 (83·6)	9·93 (13·0)
S3	4·14 (21·3)	9·1 (64·1)	4·33 (10·3)
S4	4·61 (29·7)	11·0 (80·3)	2·10 (7·6)

Table 3. *Concentrations of free amino and imino acids of* Convallaria *and* Polygonatum *tissues at different stages of growth*

Results are expressed as μg. of nitrogen present in each acid/g. dry wt. of tissue. Figures in parentheses show nitrogen present in azetidine-2-carboxylic acid expressed as percentages of total soluble nitrogen.

Sample	Asparagine	Glutamic acid	Glutamine	Azetidine-2-carboxylic acid	Serine	Arginine	Alanine
			Convallaria				
Root:							
S1	160	135	44	289 (6·2)	38	115	30
S2	223	177	93	132 (1·3)	27	561	44
S3	348	129	154	173 (2·7)	50	442	67
S4	136	129	66	653 (23·4)	46	229	74
Shoot:							
S1	150	75	56	1470 (27·6)	40	149	38
S2	443	406	782	5040 (39·7)	200	34	237
S3	292	165	94	2290 (42·4)	70	15	99
S4	17	25	6	578 (36·3)	17	25	61
Rhizome:							
S1	241	131	56	314 (6·9)	41	231	28
S2	195	61	99	207 (3·1)	49	258	7
S3	484	156	113	180 (2·5)	62	518	84
S4	100	22	60	170 (5·2)	16	368	56
			Polygonatum				
Root:							
S1	82	239	166	734 (13·0)	94	127	62
S2	73	187	98	727 (18·6)	43	91	30
S3	113	189	85	677 (16·4)	39	239	41
S4	38	151	175	2260 (49·0)	47	157	96
Shoot:							
S1	81	276	407	5580 (41·9)	115	335	55
S2	27	333	243	2260 (22·8)	107	201	107
S3	8	253	92	810 (18·7)	124	291	78
S4	13	25	13	291 (13·8)	20	25	80
Rhizome:							
S1	296	249	461	4870 (36·9)	204	247	157
S2	205	117	363	9060 (46·5)	41	619	33
S3	116	167	214	5230 (57·4)	65	338	59
S4	142	238	767	7510 (68·3)	168	498	201

observed only in the shoots. In *Polygonatum* the concentrations of most amino and imino acids fell progressively during development from S1 to S4. In *Convallaria* an initial increase generally occurred in amino acid concentrations during the early growth of the shoot (S1 to S2); further growth was accompanied by decreased concentrations of most acids.

Azetidine-2-carboxylic acid was the most important constituent of the soluble-nitrogen fraction of all *Polygonatum* tissues, but in *Convallaria* it accumulated in very high concentrations only in the shoot. Arginine and the amides, glutamine and asparagine, were also important components of the soluble-nitrogen fraction of the plants at certain stages of development.

DISCUSSION

Analytical data of the present type can be presented in several ways; the amount of any single nitrogenous component can be expressed in terms of the plant's fresh weight, dry weight or total nitrogen content. It is not easy to decide which mode of expression is most useful. Values on a fresh-weight basis are valuable for most tissues in that they reflect the actual concentrations at which the nitrogen compounds are found within the cells. However, these values have less significance for tissues like rhizome where the dry-weight/fresh-weight ratio was relatively high. They also suffer from the disadvantage that the water content and hence the fresh weight of plant tissues may vary with changes in the plant's environmental conditions to a greater extent than the dry weight. A compromise was therefore used, all values being presented on a dry-weight basis and the dry-weight/fresh-weight ratios for all tissues were included in the tables. Values can be converted into a fresh-weight basis if this is desirable.

The fact that growth of both species beyond S2 was accompanied by progressively lower total

nitrogen contents agrees with similar observations made for many other species and several factors are probably operative in producing these decreases. During shoot growth between S1 and S2 the cells of the originally underground buds must have undergone a transition from a slow to a rapid state of division and this change was associated with a lowered dry-weight/fresh-weight ratio regarded as typical of meristematic tissue having a high rate of protein synthesis. Cell division after S2 would be restricted to a smaller proportion of the cells of the shoot and the further growth to stages S3 and S4 was accompanied by progressively increased dry-weight/fresh-weight ratios, indicating that secondary thickening of many cell walls was occurring and producing increased carbohydrate to protein ratios. Decreases in the total nitrogen contents of the rhizomes and roots may in part be due to similar but less extensive changes in cellular activity in these organs. An extensive deposition, particularly in the rhizome storage tissue, of reserve carbohydrate products of photosynthesis would be expected to occur during the later part of the growing period and so this would be another factor leading to decreased nitrogen concentrations in the underground organs.

The decreases in the soluble-nitrogen concentration in the shoots between S2 and S4 are particularly striking. During this time the dry weight of the *Convallaria* shoot increased about 17-fold, whereas the total amount of nitrogen in the shoot increased only about six times. These figures may indicate that the supply of nitrogen to the shoot became limiting in the later stages of its growth and under these conditions there would be a tendency for available nitrogen to be incorporated into protein and so produce the observed marked decrease in soluble nitrogen compounds.

The exact reasons for the high concentrations of soluble nitrogen in the root and shoot of *Convallaria* at S2 must be looked for in the individual free amino and imino acid concentrations (Table 3). Although the percentage increase for some of them (glutamic acid, glutamine and alanine) was larger than that of azetidine-2-carboxylic acid, it was the increase in the total amount of the imino acid that had a profound effect on the soluble-nitrogen level. Between S1 and S2 the soluble nitrogen increased by 7·3 mg./g. dry wt.; the increased concentration of azetidine-2-carboxylic acid accounted for about half of this (3·6 mg./g. dry wt.). In contrast, the increased soluble-nitrogen levels of the root at S2 could not be attributed to changes of concentrations of azetidine-2-carboxylic acid, for although the concentrations of most other amino acids increased between S1 and S2 the imino acid levels actually decreased. Arginine showed the most marked increase of concentration and the increased amount of nitrogen present in the amino acid at S2 compared with S1 represented about one-third of the total increase of soluble nitrogen.

During the period S1–S2 the dry weight of the shoot increased 2·6 times, so that the total quantity of azetidine-2-carboxylic acid present in the shoot at S2 was approximately nine times that at S1. Although the decreases in the concentration of azetidine-2-carboxylic acid within the root and rhizome between S1 and S2 were small in comparison with the increase in the shoot, approximately half of the extra azetidine-2-carboxylic acid appearing in the shoot could have been transferred from the underground organs, since at S2 their combined weights were about 20 times that of the shoot. This calculation assumes no synthesis of azetidine-2-carboxylic acid within the root or rhizome. If a net synthesis of the imino acid was occurring in one or both of these organs, then considerably more than half of the azetidine-2-carboxylic acid appearing in the shoot may have been deposited there after translocation. A further, approximately twofold, increase in the total amount of azetidine-2-carboxylic acid in the shoot occurred between S2 and S3, for although the concentration of the imino acid decreased during this phase of growth, the dry weight of the shoot increased about fivefold. However, at this stage the concentrations of azetidine-2-carboxylic acid in root and rhizome remained nearly constant. Therefore active translocation of the compound to the shoot would appear less likely at this stage, and synthesis of the imino acid was probably occurring within the shoot itself. This conclusion is in agreement with the observation of Fowden & Bryant (1959), who showed that excised leaves of *Convallaria* at this stage of development could photosynthetically assimilate $^{14}CO_2$ slowly into azetidine-2-carboxylic acid. Little change occurred in the total amount of azetidine-2-carboxylic acid within the shoot between S3 and S4, a further decrease in its concentration being offset by an increase in the shoot dry weight. It is then perhaps significant that in this period there was a very marked increase in the concentration of azetidine-2-carboxylic acid in the root, even though the concentrations of most of the other amino acids decreased. This observation suggests that in *Convallaria* the root may act as a major site of synthesis of azetidine-2-carboxylic acid, a fact that became apparent only at this late stage of growth when active translocation of the imino acid appeared to have ceased.

Reference to Table 3 shows that in *Polygonatum* azetidine-2-carboxylic acid was the major component of the soluble-nitrogen fractions of all tissues at all stages of growth. Particularly in the rhizome variations in the concentrations of other free amino acids were completely overshadowed by

the much larger changes in concentration of azetidine-2-carboxylic acid. The imino acid represented a higher proportion of the soluble-nitrogen fraction at S4 than at any earlier stage of growth, but it reached its highest concentration on a dry-weight basis at S2, when about 6·5% of the dry weight of the rhizome consisted of azetidine-2-carboxylic acid. This is a remarkably high concentration for a soluble-nitrogen compound; comparable concentrations have been observed only for certain alkaloids (see James, 1950). A further unusual finding was that the nitrogen present in azetidine-2-carboxylic acid of the rhizome generally exceeded that present in the protein. For instance at S4 the nitrogen of azetidine-2-carboxylic acid represented about 55% and protein nitrogen only about 20% of the total nitrogen.

Decreases in the concentrations of most amino acids occurred during the growth of the *Polygonatum* shoot, particularly between stages S3 and S4. The concentration of azetidine-2-carboxylic acid at S4 was only about 5% of that at S1. Since the increase in the dry weight of the shoot during the whole period of growth was only slightly greater than 20-fold no significant increase in the total amount of azetidine-2-carboxylic acid in the shoot occurred. The possibility that active translocation of the imino acid from the underground organs to the shoot occurred therefore appeared less likely than in *Convallaria*; however, it cannot be completely ruled out since shoots of *Polygonatum* may be able to degrade the compound. Information is lacking about this degradation in *Polygonatum* but preliminary studies with [^{14}C]azetidine-2-carboxylic acid indicated that degradation proceeded very slowly in *Convallaria* leaves (Fowden & Bryant, 1959).

Attention was drawn earlier to the increase in concentration of azetidine-2-carboxylic acid in *Convallaria* roots between S3 and S4. Table 3 shows that a similar rapid increase occurred for the imino acid in *Polygonatum* roots. Although some significance was attached to this finding in regard to the site of synthesis of azetidine-2-carboxylic acid in *Convallaria*, the position in *Polygonatum* is more obscure. The increase in concentration of azetidine-2-carboxylic acid in the roots was paralleled by an even larger increase in the amount of the imino acid present in the rhizome. Therefore the possibility that synthesis can proceed in both organs cannot be overlooked. Fowden & Bryant (1959) found that radioactivity supplied to roots and rhizome of *Polygonatum* in the form of [^{14}C]aspartic acid was incorporated into azetidine-2-carboxylic acid more rapidly in the former organ, although some azetidine-2-carboxylic acid was apparently also synthesized in the rhizome.

The role of azetidine-2-carboxylic acid in the nitrogen metabolism of liliaceous plants remains in doubt. The high concentrations in which it is encountered suggests that it is more likely to play the role of a storage compound rather than that of an actively metabolized intermediate compound. This conclusion receives some support from experiments with [^{14}C]amino acids, which showed that the rates of synthesis and degradation of the imino acid in these plants were slow (Fowden & Bryant, 1959). If azetidine-2-carboxylic acid is correctly regarded as a storage compound, then nitrogen so combined is probably less readily available for re-utilization than that present in other compounds such as glutamine and asparagine that are considered to play a similar role. The high concentrations and relatively low metabolic activity of azetidine-2-carboxylic acid in plants suggest that it may be more closely akin to the alkaloids than to the majority of amino acids. Indeed it is tempting to speculate that alkaloid-like compounds based on the four-atom heterocyclic ring of azetidine will ultimately be discovered in plants. A compound bearing the same structural relation to azetidine-2-carboxylic acid as that shown to proline by the alkaloid stachydrin would seem a distinct possibility.

If the idea is accepted that azetidine-2-carboxylic acid is synthesized largely in the root of *Convallaria* and then translocated and deposited as a storage compound in the leaves, a further similarity with certain alkaloids emerges. For example, a parallel exists with nicotine, which is elaborated by the roots of the tobacco plant (Mothes, 1928; Dawson, 1941, 1942). However, nicotine tends to be present in this organ in only low concentrations and is mainly transported to the leaves, where it is stored at much higher concentrations.

Our present understanding of the behaviour and function of azetidine-2-carboxylic acid in plants is certainly limited. The compound possesses features of metabolism which identify it closely in behaviour with a group of other compounds like alkaloids, floral pigments, glycosides, etc., the reason for whose existence in plants still remains unsolved.

SUMMARY

1. Variations occurring in the nitrogenous composition during the growth of *Convallaria majalis* and *Polygonatum multiflorum* plants were investigated. At four stages of growth the concentrations of total nitrogen, 75% (v/v) ethanol-soluble nitrogen and certain amino and imino acids were determined separately for roots, rhizome and shoots.

2. The soluble-nitrogen content of both plants, but particularly that of *Polygonatum*, was high

when expressed as a percentage of the total nitrogen. This observation is in agreement with those recorded previously for typical storage organs.

3. The two plants contained very large amounts of the imino acid, azetidine-2-carboxylic acid. Nitrogen present in azetidine-2-carboxylic acid represented more than 50% of the total nitrogen of *Polygonatum* rhizome. The imino acid was also present in high concentrations in the young shoots of both plants, but its concentration in the mature shoots was much lower.

4. The analytical data indicate that the root may form a major site of synthesis of azetidine-2-carboxylic acid in *Convallaria*. It is more difficult to draw conclusions for *Polygonatum* since the very high concentrations present in its rhizome at all stages of growth tended to overshadow smaller changes of concentration elsewhere in the plant.

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Nitrogenous Constituents of the Thoracic Muscle of the African Migratory Locust (*Locusta migratoria migratorioides*)

By W. O. KERMACK and JANET M. STEIN
Department of Biological Chemistry, University of Aberdeen

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During recent years a considerable amount of information has become available concerning the non-protein nitrogenous constituents, and especially the amino acids in the haemolymph of various species of insects. Most of this is due to the work of Florkin and his colleagues, who have in the main used microbiological methods for their assays of amino acids (Florkin, 1954). It appears that the haemolymph of insects is characterized by a relatively high amino acid content, and in this respect they differ markedly from crustaceans such as lobsters which, though containing a high concentration of free amino acids in their muscles, have very little in the haemolymph (Kermack, Lees & Wood, 1955). Little or no information appeared to be available about the free amino acid content of insect muscle.

An insect which it seemed convenient to study in this respect was the migratory locust *Locusta migratoria migratorioides*. The insect order Orthoptera, to which the locust belongs, contains many of the larger insects, and a considerable amount of research has been carried out on this order. From the available data, the metabolism of orthopteran muscle appears to be similar to that of mammalian muscle; for example, the occurrence of glycolysis and the presence of the tricarboxylic acid cycle in the African migratory locust has been demonstrated by Rees (1954) and adenosine triphosphate has been isolated from the muscle of the locust *Gastrimargus musicus* (Calaby, 1951).

In the present work the nature of the non-protein nitrogenous constituents of locust muscle was investigated. Some of the present results have already been briefly presented (Kermack & Stein, 1955).

MATERIALS AND METHODS

Locusts. Fifth-stage nymphs of the African migratory locust, supplied by the Anti-Locust Research Centre, London, were kept in the Laboratory under the conditions advised by the Centre. The locusts, after undergoing their final moult to the adult form, were used as required. After starving overnight they were killed by placing for a few minutes in a jar containing chloroform, then cut open dorsally, the alimentary canal and fat body were removed and the thoracic muscles *in situ* washed free of haemolymph by squeezing 1% (w/v) NaCl from a Pasteur pipette between and underneath the muscle bands. Residual saline on the surface was removed with filter paper and the intact muscle excised.

Muscle extracts. Muscle from about six locusts was homogenized with 5% (w/v) trichloroacetic acid (5 ml.) for 5 min. in a Mickel homogenizer. The suspension was allowed to stand for 15 min. and centrifuged. The muscle residue was extracted a second time in the same way, and

the combined supernatants were made up to 20 ml. with water. Ethanol extracts of muscle were made similarly, ethanol being substituted for 5% trichloroacetic acid.

Muscle hydrolysates. The thoracic muscle from one locust was hydrolysed with 6N-HCl in a sealed glass tube at 110° for 16 hr. Larger amounts were prepared by refluxing the muscle of a number of locusts with 6N-HCl for 24 hr. HCl was removed by distillation under reduced pressure.

Perchloric acid hydrolysis of muscle extracts. A volume (10 ml.) of locust extract was evaporated to dryness at 100°; 0·5 ml. of 60% (v/v) perchloric acid was added and the mixture kept at 100° for 1 hr.

Total non-protein nitrogen determinations. Trichloroacetic acid extract (1 ml.) of locust muscle was digested by the method of Chibnall, Rees & Williams (1943). Distillation was carried out in the apparatus described by Markham (1942) and the ammonia collected in 1% (w/v) boric acid containing Conway indicator (Conway, 1950).

α-Amino nitrogen. This was determined by the method of Pope & Stevens (1939).

Uric acid. This was determined by the method of Folin (1934).

Paper chromatography. Whatman no. 1 paper was used for general work, and Whatman no. 4 for quantitative work. For a two-dimensional paper chromatogram, 100–150 μl. of locust extract, containing 5–10 μg. of α-amino N, was applied to the paper from a micropipette. Solvents used were phenol–water (4:1, v/v) and butan-1-ol–acetic acid–water (4:1:5, by vol.) (Partridge, 1948). Amino acids were located by spraying the paper with ninhydrin in the usual way. In addition the identities of the following amino acids were checked by spraying chromatograms with the appropriate specific reagent: arginine (Block, LeStrange & Zweig, 1952); histidine and tyrosine (Block, 1949); proline and hydroxyproline (Acher, Fromageot & Jutisz, 1950); methionine, cysteine and cystine (Toennies & Kolb, 1951).

Quantitative determination of amino acids. A modification of the method of Fowden (1951) was used. The location of amino acids on the chromatogram by their fluorescence in ultraviolet light, recommended by Fowden, was found to be incapable of detecting some of the amino acids present in low concentrations. Amino acids were located by spraying the chromatogram with a weak solution (0·05%, w/v) of ninhydrin in butanol (Landua & Awapara, 1949) and allowing it to dry at room temperature overnight. Ammonia was rigorously excluded from reagents and chromatographic paper throughout the work. The faint spots were cut out from the chromatogram and a paper blank was cut out, of the same area. The spots were placed in a series of small tubes, 1 ml. of water was added, and left overnight. A volume (1 ml.) of the ninhydrin stock solution was then added and the procedure completed as described by Fowden (1951).

Proline was estimated approximately by spraying one-dimensional chromatograms of locust extract with isatin reagent (Acher *et al.* 1950), and comparing the size and intensity of the proline spot with standard spots on parallel chromatograms.

Purines. These were investigated with one-dimensional paper chromatography in propan-2-ol–HCl (Wyatt, 1951), and the positions of compounds absorbing in the ultraviolet were located by the photoprint method of Markham & Smith (1949). Quantitative determinations of the purines were carried out by the method of Wyatt (1951).

Phosphate. Phosphate present in purine nucleotides eluted from the chromatograms was determined by the method of Berenblum & Chain (1938) as modified by Long (1943).

RESULTS

Determinations of total non-protein nitrogen

Determinations of non-protein N were carried out on trichloroacetic acid extracts prepared from batches of locusts of different ages, including one group of females and one group of males dissected immediately after the final moult. The non-protein N values of the muscle extracts are given in Table 1.

The non-protein N values range between 300 and 400 mg./100 g. of muscle, and show no obvious correlation with either the age or the sex of the locusts. The variation may be due partly to experimental error and partly to the fact that the figures are drawn from analyses of muscle extracts from different stocks of locusts. Table 2 gives the results of four determinations of non-protein N on extracts from the females of a single stock of locusts. The figures indicate that the non-protein N content of the muscle extracts increased over the first 10 days after the moult and subsequently became relatively constant.

Free amino acids

The amino acids were detected in trichloroacetic and ethanolic extracts of muscle by two-dimensional paper chromatography. The relative intensities of the ninhydrin colour of the various spots are indicated in Table 3.

Table 1. *Total non-protein nitrogen values of trichloroacetic acid extracts of muscle*

Time after final moult (days)	Total non-protein N (mg./100 g. of fresh muscle)	
	Females	Males
0	326	310
15–18	—	398
17–20	350	—
18–22	370	368
21–28	320	340
25–28	327	—
35–38	328	329
35–42	356	355
65	299	315

Table 2. *Total non-protein nitrogen values of trichloroacetic acid extracts of muscle from a single stock of female locusts*

Time after final moult (days)	Total non-protein N (mg./100 g. of fresh muscle)
0–2	288
11	310
20	336
30	336

Table 3. *Semi-quantitative estimation of free and protein-bound amino acids in locust muscle*

–, Signifies not detectable.

	Trichloroacetic acid extracts of muscle	Ethanolic extracts of muscle	Muscle hydrolysate
α-Alanine	+ + +	+ +	+ +
β-Alanine	–	+	–
Arginine	+ + + +	+	+ +
Aspartic acid	+	–	+ + +
Cysteic acid	Trace	–	Trace
Glutamic acid	+	+	+ + +
Glutamine	+ + + +	+ + +	–
Glycine	+ +	+ + +	+ +
Histidine	–	+	+
Leucine (including isoleucine)	+ + +	+	+ +
Lysine	Trace	+	+ +
Methionine sulphoxide	–	–	Trace
Phenylalanine	–	+	+ + + +
Proline	+ +	+	+
Serine	+	+	+ +
Taurine	+ + + +	+ + +	–
Threonine	–	+	+ +
Tyrosine	Trace	+	+
Valine	+	+ +	+ +

Table 4. *α-Amino nitrogen values of trichloroacetic acid extracts of muscle*

Time after final moult (days)	α-Amino N (mg./100 g. of fresh muscle)
Females	
0	104
1–2	118
7–10	194
17–20	111
18–22	94
21–28	86
25–28	77
35–38	114
65	77
Males	
0	92
8–11	176
15–18	93·5
18–22	88
24–27	83
21–28	87
36–39	105
65	71

Determinations of total α-amino nitrogen

The results obtained with trichloroacetic acid extracts are given in Table 4. It can be seen from these figures that the α-amino N values differ between the sexes, the value for female locusts being usually higher than for males of the same age. The α-amino N also varies with the age of the insect, rising from a low value immediately after the final moult to a peak and then falls. In females this fall is usually found to occur about 3 weeks after the final moult and may correspond with egg formation. In males the fall occurs about 2 weeks after the final moult and may correspond with sperm formation. After this fall values remain low for about 2 weeks, during which time mating is at its height (Norris, 1950). Later, the α-amino N rises slightly, but falls off as the locusts become older and more sluggish.

The possibility that the changes in the α-amino N content of tissue extracts were related to the reproductive activity of the locusts was investigated further. A series of extracts were prepared from the females of two batches of locusts, and the state of the ovaries and the time at which egg-laying began were noted. The first batch, from which extracts 35, 36, 37 and 38 were prepared, was found to be parasitized with the nematode worm *Mermis nigrescens* and no egg-laying had occurred. The second batch, from which extracts 40, 41 and 42 were prepared, began egg-laying later than usual, owing to the scarcity of fresh grass for their food. Locusts in extract 40 had ovaries that were not fully developed, whereas in locusts in extracts 41 and 42 the ovaries were well developed, and egg-laying was intense in the period between the making of the two extracts. It would appear from the values given in Table 5 that the α-amino N was high when the eggs were being formed (extract 41) and fell after the eggs had been released (extract 42).

Determinations of individual amino acids

The aim of these investigations was to study the effects, if any, of egg-laying on the amounts of the individual amino acids present. The results of a number of estimations carried out on extracts from female locusts are presented in Table 5. As stated above, extracts 35, 36 and 37 show the effect of increasing age, uncomplicated by egg-formation.

Table 5. *Quantitative estimation of individual amino acids in trichloroacetic acid extracts of muscle of female locusts*

Amino acid values are given as mg. of α-amino N/100 g. of muscle.

Extract no.	35	36	37	38	40	41	42
Days after final moult	0–2	11	20	30	10	35	44
α-Amino N (Pope & Stevens, 1939)	60·5	91	83	89·5	75	98·5	71
Aspartic acid	—	0·1	1·5	—	—	—	—
Glutamic acid	—	1·5	2·7	—	—	1·8	0·5
'Spot 1'	—	3·0	4·2	—	—	3·0	1·0
Glycine	—	12·9	6·4	—	—	28·0	31·0
Alanine	3·5	15·5	15·8	—	—	12·2	4·6
Valine	1·8	2·7	3·8	—	—	1·3	0·5
Leucine (including isoleucine)	15·0	13·4	12·9	—	—	2·8	0·5
Glutamine	17·3	13·9	20·7	—	—	12·1	1·3
Arginine	23·0	20·0	22·8	—	—	15·6	23·8
Serine	—	—	2·6	—	—	—	—
Proline	6·2	7·4	10·2	—	—	18·8	13·2
Taurine N	9·3	15·5	36·4	47	—	30·0	33·5

Table 6. *Uric acid nitrogen content of trichloroacetic acid extracts of muscle*

Time after final moult (days)	Uric acid N (mg./100 g. of muscle)
Females	
0	8·2
5–7	1·5
35–42	<1·3
Males	
0	8·3
35–42	<1·7

Table 7. *Estimation of adenylic acid in locusts of different ages*

Time after final moult (days)	Adenylic acid N (mg./100 g. of fresh muscle)
0 (males)	45
5–7 (females)	41
19 (males)	56
21–28 (females)	67
35–40 (females)	60

Extracts 41 and 42 show the changes in amino acids before and after oviposition. A ninhydrin-staining spot having the same R_F as glutamic acid in phenol, and a slightly lower R_F than glutamic acid in butan-1-ol–acetic acid–water is referred to in Table 5 as 'spot 1'. Since the spot was not identified, the strength of the spot was compared with that of a glutamic acid standard, and the α-amino N content then calculated as if the unknown were glutamic acid.

The increase in α-amino N between extract 35 and extract 36 corresponds with increases in most of the amino acids, although glycine is a notable exception.

In the muscle tissue from the second batch of locusts, glutamine shows the greatest change; it falls from 12·1 mg. of α-amino N in extract 41 to 1·3 mg. of α-amino N in extract 42. Glutamic acid, 'spot 1', alanine, valine and leucine (including isoleucine) all fall to one-third or less of their original value, and proline to about two-thirds of its original value. On the other hand, glycine rises slightly in amount and arginine increases considerably. In both batches of locusts, the amount of taurine increases with the age of the locusts and is unaffected by egg-laying.

Determinations of uric acid

No uric acid was detected in extracts of muscle of male and female locusts of 35–42 days. From the sensitivity of the method used, the maximum amount of uric acid that could have been present was calculated, and is included in Table 6. It would appear from these results that only over the period of moulting does uric acid account for any appreciable amount of extractable non-protein N in muscle extracts.

Adenylic acid

A spot absorbing ultraviolet light, believed to be that of adenylic acid, was detected on chromatograms of locust-muscle extract. The maximum absorption of the eluted spot was at 260 mμ. The R_F value (0·39) of the unknown spot with the propan-2-ol–HCl solvent (Wyatt, 1951) was similar to that of adenylic acid (0·41), and after perchloric acid hydrolysis was identical with that of adenine (0·26).

The unknown spot contained phosphorus. After elution from the chromatogram the amount of adenine was determined spectrophotometrically, and the amount of phosphorus determined. The molecular proportions of adenine to phosphate were 1:1, indicating that the unknown substance was adenylic acid, rather than adenosine diphosphate or adenosine triphosphate. On the assumption that the unknown substance was adenylic acid, the results of quantitative determinations carried out on muscle extracts from several batches of locusts are given in Table 7. It is probable that the

adenylic acid found in muscle extracts is partially present as adenosine triphosphate in the living insect, but has been hydrolysed at the low pH at which the extracts were prepared and stored.

Muscle hydrolysates

The amino acids identified in muscle hydrolysates are included in Table 3, the intensity of the ninhydrin-staining spots being as indicated. Further, a spot believed to be lanthionine consistently appeared on the chromatograms, its position being identical with that of a sample of lanthionine. Evidence of the identity of this compound with lanthionine has been published previously (Stein, 1955).

Distribution of non-protein nitrogen in tissue extracts

Table 8 is a description of a typical locust-muscle extract. The volatile-amine value included is only an average value, taken from a series of estimations by the method of Conway (1950). It must be emphasized that many of the values quoted in the table are dependent on the age, sex and physiological state of the locusts.

DISCUSSION

Each muscle extract used in this work was prepared from a number of locusts, all of the same age and sex. In this way, individual variations were averaged, and the effects of age, sex and physiological conditions could be studied by comparison of different groups. A criticism of the present work might be that the muscle extracts were possibly contaminated by haemolymph contained within the intercellular spaces, since no estimation of the volume of the intercellular spaces was made. Some observations which we have made on the amino acid content of locust haemolymph suggest, in agreement with the results of Kilby & Neville (1957, personal communication), that the α-amino N content is of the order of 100 mg./100 ml. This is in agreement with the recent work of Patterson (1956), which indicates that the hemimetabolous group of insects, to which the locust belongs, has not as high a concentration of amino acids in the haemolymph as the holometabolous group, various representatives of which have been studied by Florkin (1954). Thus the concentration of amino acids in locust haemolymph is about the same as that we have found in locust tissue, and it is not possible to explain the concentration of amino acids found in the muscle on the assumption that the muscles themselves have low concentrations of amino acids and owe their relatively high concentration to contamination by haemolymph. Chromatographic investigations of the amino acids confirm this conclusion, for they show that asparagine and tyrosine are present in haemolymph, but not in muscle extracts, whereas taurine, alanine, glutamic acid and aspartic acid are present in muscle extracts, but not in haemolymph (authors' unpublished work). Thus it seems probable that there was no gross contamination of the extracts by haemolymph.

Table 8. *Distribution of nitrogen in trichloroacetic acid extracts of muscle from female locusts* 11 *days after final moult*

	N (mg./100 g. of fresh muscle)
Total N	310
α-Amino N	91
Taurine N	16
Arginine guanidyl N	68
Glutamine amide N	14
Adenylic acid N	45
Uric acid N	1
Volatile-amine N	20
Total	255

The qualitative investigations of the amino acids in locust-muscle hydrolysates suggest that locust-muscle protein is made up of a number of α-amino acids that are chromatographically indistinguishable from those found in other hydrolysed proteins. In addition, lanthionine has been detected in muscle hydrolysates, and it is probable that this amino acid exists preformed in the muscle protein of the locust, and is not merely produced as an artifact during hydrolysis (Stein, 1955).

All of the amino acids detected in hydrolysates of locust muscle also occurred in protein-free muscle extracts, except lanthionine and methionine sulphoxide. (The latter only appeared in traces and may be an artifact formed by oxidation of methionine during hydrolysis.) In addition, muscle extracts contained taurine and β-alanine. The relative proportions of the amino acids in muscle hydrolysates and in muscle extracts were quite different.

The total α-amino N content of muscle extracts was observed to vary with the physiological state of the insect. In males, a decrease in the α-amino N was observed about 2 weeks after the final moult, and possibly corresponded with the release of sperm. In females, the fall in α-amino N occurred about 3 weeks after the final moult, and corresponded with the onset of egg-laying. Cheu (1952) has found that at the time of egg-laying approximately 144 mg. of protein is lost from the locust with each egg-pod. According to Cheu, this represents about one-quarter of the total protein built up by the insect from the time of the final moult to the onset of oviposition. Since a mature female *Locusta migratoria*, fed on a normal diet, will lay

five to six egg-pods in the course of its life at the rate of one egg-pod about every 5 days (Norris, 1950), evidently there must be a rapid rate of protein synthesis throughout the reproductive period. Since the locust stops feeding just before oviposition, the level of amino acids in the tissues might be expected to fall during oviposition.

With the exception of taurine in some extracts, arginine was the most abundant amino acid. Part of it at least was presumably derived from arginine phosphate present in the intact muscle and hydrolysed during the preparation of the extract. This may be related to the fact that the amount of arginine present in the extracts was not affected by the general decrease in the level of amino acids at the time of egg-laying. Glutamine was present in considerable quantity in muscle extracts; in the absence of egg-laying the glutamine level remained fairly constant, whereas at the time of oviposition it fell to 11% of its original value. This suggests that glutamine acts as a nitrogen store which is used in the formation of the egg-pods. It can be seen from Table 5 that although the amount of glutamine in locust-muscle extracts was reduced at the time of egg-laying, the amount of glutamic acid was not correspondingly increased. It may be inferred that the glutamine which disappeared was not simply deamidated, but was incorporated into protein or otherwise metabolized. Kilby & Neville (1956) have demonstrated the presence of a glutamic dehydrogenase in the fat body, and of glutamate–alanine and glutamate–aspartate transaminases in the fat body, Malpighian tubules and gut wall of the desert locust. It is probable that these enzymes are also present in the African migratory locust, and may account for the disappearance of the glutamic acid produced by the glutamine.

In many muscle extracts, taurine was the most abundant amino acid, and the amount increased with the increasing age of each batch of locusts. Taurine also occurs in lobster muscle in amounts comparable with those found in locust muscle (Kermack *et al.* 1955), and is present in the muscle of *Mytilus edulis* (Baldwin, 1952).

The uric acid content of locust-muscle extracts was greatest immediately after the final moult. This may be due to the temporary cessation of the excretory processes at ecdysis, when uric acid formation is probably increased. The amount present declined with the increasing age of the locusts, and none could be detected 35 days after the final moult.

The adenylic acid detected in locust-muscle extracts possibly in part represented adenosine triphosphate in the intact muscle which had been hydrolysed as a result of the acid conditions under which the extracts were prepared and stored. The amount of adenylic acid in locust muscle was of the same order of magnitude as the amount (present in the form of adenosine triphosphate) in mammalian skeletal muscle. The rise in concentration of adenylic acid during the first weeks of the locusts' adult life would be presumably correlated with their gradually increasing muscular power.

SUMMARY

1. Total non-protein nitrogen values of trichloroacetic acid extracts of locust muscle varied from 290 to 400 mg./100 g. of muscle and rose with the increasing age of the locusts. About 80% of the total non-protein nitrogen was accounted for as amino acid nitrogen, purines and volatile amines.

2. α-Amino nitrogen values of trichloroacetic acid extracts of muscle varied from 60 to 190 mg./100 g. of fresh muscle. The values were low in the extracts made from the muscle of locusts shortly after emergence from the final moult, but rose to a peak before the onset of mating.

3. In trichloroacetic acid extracts of muscle the amount of taurine increased with the increasing age of the locusts. Glutamine was abundant before egg-laying and fell to one-tenth of its original value after this time. Glutamic acid, alanine, valine, leucine (including isoleucine) and an unidentified ninhydrin-staining substance all fell to about one-third of their original amounts at the time of egg-laying, whereas arginine and glycine rose slightly.

4. Uric acid accounted for 8 mg. of nitrogen/100 g. of muscle in newly moulted adult locusts. The value fell to 1·5 mg. 6 days later, and none could be detected after 14 days.

5. Adenylic acid accounted for 20–60 mg. of nitrogen/100 g. of fresh muscle, and was presumably present as adenosine triphosphate in the intact muscle.

6. Seventeen amino acids were detected in acid hydrolysates of locust-muscle protein, including the unusual amino acid lanthionine.

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γ-Amino-*n*-Butyric, Aspartic, Glutamic and Pyrrolidonecarboxylic Acid; Their Determination and Occurrence in Grass during Conservation

By H. T. MACPHERSON and J. S. SLATER
Chemistry Department, The King's Buildings, Edinburgh University

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The harvesting of a forage crop is followed by rapid and extensive proteolysis, terminated by the attainment of a high dry matter or a low pH as the material is dried or ensiled respectively. Even in a good silage about 50% net breakdown of the protein may occur; the proteolysis on drying is smaller, but where drying is slow it may be considerable. Since the fresh crop contains 80–90% of its nitrogen in the form of protein, large amounts of amino acids are released and it is of interest to know what happens to them. It was evident from preliminary analyses of wilted grass and silage extracts that the amounts of aspartic and glutamic acid, which together account for nearly one-seventh of the protein, fell very far short of the amounts expected from the resulting proteolysis. However, other closely related substances, namely asparagine, glutamine, pyrrolidonecarboxylic acid and γ-amino-*n*-butyric acid, were present in the conservation product, often in considerable quantity, and it was desirable that they should be estimated with some accuracy.

For the aspartic and glutamic acid themselves, a rapid single-stage ion-exchange method based on that of Hirs, Moore & Stein (1954) has been developed. Pyrrolidonecarboxylic acid, for which no simple method appears to exist, was determined on the same sample. The method of Vickery, Pucher, Clark, Chibnall & Westall (1935) appeared to be satisfactory for the amides, particularly when present in quantity, and they can be checked as pyrrolidonecarboxylic acid and aspartic acid. A new and simple method for γ-aminobutyric acid determination has also been evolved. These methods have been applied to proteins, to fresh grass and to grass kept under various conditions encountered in conservation practice. From the results obtained, some general conclusions have been drawn about the metabolism of these substances during conservation.

METHODS

Material for analysis. In all the analyses given below, extraction has been carried out on undried material as soon as possible after sampling. Very often, particularly where a large number of samples is involved, drying would be a convenient method of preparation for storage, and sampling errors are probably reduced on milled samples. However, while there is little or no loss of nitrogen on oven-drying, except where volatile-base values are high, as in some silages, changes of nitrogenous and other substances may be considerable and some sort of forced-draught drying is preferable. A much-enhanced soluble-nitrogen figure may be obtained when elevated temperatures are employed without rapid dissipation of moisture, owing to increased rate of enzyme action.

Extraction of material for analysis. We have generally employed hot-water extraction for obtaining protein-free, soluble-N fractions. Boiling water is poured on the fresh sample, which is then macerated or kneaded for a few minutes, and the liquid poured off and cooled rapidly. This is done three times and the combined extracts are concentrated *in vacuo* to the required volume; the solution is then filtered and kept under toluene at 0°. A precipitate may separate subsequently, but this does not appear to contain any nitrogen. The main objection to the method is that the use of heat tends to cause some conversion of glutamine into pyrrolidonecarboxylic acid and ammonia, but the time of heating is short and the small amount of pyrrolidonecarboxylic acid is in any case determined. To obtain a true analysis of these substances, determinations of volatile base, glutamine, glutamic acid and pyrrolidonecarboxylic acid should all be done as soon as possible.

Determination of glutamine and asparagine. These are determined by the methods of Vickery *et al.* (1935), both being measured by the extra ammonia formed on hydrolysis: glutamine for 2 hr. at pH 6·5 and asparagine for 3 hr. in N-H_2SO_4, both at 100°.

Determination of glutamic and aspartic acid. Wiltshire (1953) has described a separation by the two-column method of Consden, Gordon & Martin (1948) followed by Van Slyke amino N determination. Hirs *et al.* (1954) have used a Dowex-1 acetate column but report poor recoveries, and Campbell (1956) has investigated the separation of dicarboxylic from other amino acids using acetic acid and resins of varying cross-linking. The method described here employs a single short column of De-Acidite FF in the acetate form eluted with dilute acetic acid at a comparatively rapid flow-rate, the determination of the acids being completed in about 4 hr. The method has proved thoroughly reliable in a very large number of analyses.

Resin of 90–120 mesh is obtained from the coarser 16–50 particles by breaking down for a few minutes in a ball mill and selecting the appropriate fraction; this does not appear to affect the functioning of the resin. The resin is nominally 3·5% cross-linked. It is washed with N-NaOH in a conical flask, then thrice water-washed, with settling and decanting each time. After washing once with 20% (v/v) acetic acid and twice with water it is finally stored under 1% (v/v) acetic acid. Enough to fill several columns should be prepared. Regeneration of the used material is carried out in the same way and the useful life appears to be considerable. A 15 cm. depth is poured from the 1% acetic acid suspension into a 1·2 cm. column with a very small cotton-wool plug in the bottom. The column is tapped on the bench to ensure close settling, the sides are washed with 1% acetic acid and a small glass-wool plug is firmly tamped on top. Finally, 4–5 bed-volumes of 0·1% acetic acid are run through and the column is ready for use; it should be used immediately.

The sample of extract, 2–3 ml. containing up to 2 mg. of N, is put on with a bent-tip pipette and allowed to drain in slowly, being washed in with 1–2 ml. of 0·1% acetic acid. Separation of the dicarboxylic amino acids from other ninhydrin-reacting substances and from each other can be varied by changes in strength of the eluting acid and by promotion or postponement of such changes. The following procedure was satisfactory. When the sample has been washed on, 1% acetic acid is applied to the column at 40–45 ml./hr. When a total of about 30 ml., including sample and washing effluent, has been collected, the solvent is changed to 3% (v/v) acetic acid flowing at a similar rate and a further 80 ml. or so is collected. A fraction-collector may be used, but in most of this work fractions were collected via a siphon delivering 3·2 ml. (±0·1 ml.) into 6 in. × ⅝ in. tubes graduated at 10 ml. Usually 25 fractions are collected after the change to 3% acid and the position of the peaks is very consistent, glutamic acid coming off in a very sharp band at the seventh and eighth fractions, followed closely by the aspartic band. The amino acids are then measured by ninhydrin colorimetry, 2 ml. of the Moore & Stein (1954) strongly buffered ninhydrin–hydrindantin reagent being added to the contents of each tube. The tubes are immersed in boiling water for 20 min., cooled to room temperature, made to volume with 50% ethanol and shaken vigorously for a few seconds; the intensities are then read after about 20 min. Comparison is made with an appropriate series of glutamic and aspartic acid standards freshly made in 3% (v/v) acetic acid and developed at the same time, which eliminates the need for stringent precautions in the preparation of the reagent. As a refinement, the standards are read against a blank of acetic acid and reagent, and the column fractions against a column blank, plus reagent, taken after the aspartic acid has emerged; generally the blanks are almost identical. As long as a fresh column is used, the high blanks obtained by the use of HCl (Wiltshire, 1953) are not found. Usually, however, the blank preceding the glutamic acid peak is greater than that following the peak and this may be due to the fact that, even with the strongly buffered reagent, the pH in the presence of 1% acetic acid is slightly higher than with 3% acetic acid and a slightly deeper colour results.

Determination of pyrrolidonecarboxylic acid. By an extension of the above method, pyrrolidonecarboxylic acid can also be determined as glutamic acid. After the fractions required above have been collected, the eluent is changed to 5% (v/v) formic acid and 50 ml. run through. The eluate is taken to dryness on the water bath and a few millilitres of 6N-HCl are added: the vessel is covered with a watch-glass and heating is continued for 1·5 hr., the glass then being removed and the solution evaporated to dryness. This treatment has been shown to be sufficient for complete conversion into glutamic acid. The residue is taken up in a little cold water and the glutamic acid determined on a fresh column as described above. Ninhydrin colorimetry cannot be applied directly to the residue because the HCl treatment gives rise to amine from column residues appearing in the formic acid eluate; this basic material comes off early on the second column and a clean glutamic acid fraction is obtained.

Determination of γ-aminobutyric acid. Waksman & Bigwood (1955) have described a separation of the acid in a long buffered column of Dowex 50, but the procedure now described is quicker, and uses a volatile solvent. The sample is applied to a short column of cation-exchanger in the pyridine form and the elution is performed with weak aqueous pyridine. Total time occupied in separation and determination is about 5 hr. The resin used is Zeo-Karb 225, 4·5% cross-linked, 200–400 mesh (The Permutit Co. Ltd.). Preliminary treatment followed closely that of Moore & Stein (1951) for Dowex 50, except that selection of particles by sieving was omitted. An amount (1 lb.) of the material as supplied was suspended in 2 l. of 4N-HCl and allowed to settle for 15 min., the supernatant being discarded; after three such treatments the resin was washed with water and suspended in 2N-NaOH. The suspension was heated on a boiling-water bath with occasional stirring and replacement of the NaOH until the supernatant was clear and colourless (four or five changes). The resin was again washed with water, suspended once in 2N-HCl, washed till neutral and finally shaken up and stored in 5% (v/v) pyridine.

Some of the stock resin prepared as above is suspended in 0·2% (v/v) pyridine and a column 15 cm. × 1·2 cm. poured. Two bed-volumes of 0·2% pyridine are then passed through and the column is ready for use. With columns prepared from a new batch of resin the flow-rate under a 40 cm. head is about 20 ml./hr., increasing to about 30 ml./hr. after several regenerations owing to loss of the finer particles; however, this has no apparent effect on the position of the peaks. Regeneration is carried out by

heating twice with NaOH on the bath, then the subsequent treatment is that described above.

A small sample of extract containing up to 2 mg. of total N is applied to the top of the resin and washed in with a little 0·2% (v/v) pyridine. The column is then filled up with 0·2% pyridine, a reservoir fitted and 2 ml. fractions are collected. The γ-aminobutyric acid band generally appears in fractions 33–43 with maximum at 37. The ninhydrin colour is developed and measured against standards as described for the dicarboxylic amino acids. The column and reagent blanks may differ slightly unless the pyridine used in the standards has been run through Zeo-Karb 225, H^+ form, to remove traces of a ninhydrin-reacting impurity.

Cellosolve blanks. Some batches of ethyl Cellosolve (2-ethoxyethanol), which we have generally employed instead of methyl Cellosolve (2-methoxyethanol), show a considerable ninhydrin blank. This can be removed readily by running it through a column of Zeo-Karb 215, H^+ form, previously washed through with Cellosolve to remove traces of yellow colour from the resin eluate.

In a typical aminodicarboxylic acid separation, the last of the amino acids to appear before glutamic is tryptophan, which is not separated from glutamic acid on the column of Hirs *et al.* (1954) but is well separated here, preceded by tyrosine and phenylalanine. In the separation of γ-aminobutyric acid, the first peak contains aspartic and glutamic acid, the second the monoaminomonocarboxylic acids, the third and fourth phenylalanine and tyrosine respectively and the fifth γ-aminobutyric acid. Tryptophan comes off much later with stronger pyridine. Separation of the early peaks can be improved on a longer column. β-Alanine overlaps phenylalanine and δ-aminovaleric acid appears earlier; glucosamine and ethanolamine were not found in the 0·2% pyridine eluate.

Recoveries. Recovery of glutamic acid from the De-Acidite column is 100±1% and that of aspartic acid 99±1%. In the present instance there appears to be no need to correct for 'destruction' of glutamic acid, as is often done when buffered columns are employed. Glutamic acid left on the column for 24 hr. before elution has been recovered quantitatively. The recovery of aspartic acid is nearly always just less than 100% and very faint traces are sometimes found in the formic acid eluate. No other ninhydrin-reacting substance was found on chromatography of the glutamic and aspartic acid bands, either before or after hydrolysis.

Recovery of pyrrolidonecarboxylic acid by the method described has been tested on an authentic sample containing 7% of free glutamic acid. The free glutamic acid was determined directly in the sample after chromatography, the residual pyrrolidonecarboxylic acid being recovered quantitatively as glutamic acid from the formic acid eluate. The method for pyrrolidonecarboxylic acid is valid as long as (*a*) all the glutamic acid is eluted by 3% (v/v) acetic acid and (*b*) no other glutamic acid- or glutamine-containing material is present in the formic acid eluate. The first point has been checked by heavily loading a column with glutamic acid, and the second by running paper chromatograms of the formic acid fractions. The presence of pyrrolidonecarboxylic acid was demonstrated by the appearance of an acid spot with the correct R_F, hydrolysis of which yielded only glutamic acid. The remainder of the chromatograms showed no trace of glutamic acid free or combined, though very faint spots of other amino acids sometimes appeared after hydrolysis. However, since pyrrolidonecarboxylic acid is determined as glutamic acid on a fresh column, these do not affect the determination. In the paper chromatography of the pyrrolidonecarboxylic acid fraction the formic acid eluate was dried *in vacuo* in the cold to prevent hydrolysis.

Five experiments on two complete amino acid mixtures gave recoveries of 97·5–103·6% (mean 100·1) of the γ-aminobutyric acid. Three experiments in which known amounts were added to a fresh grass extract of small, known γ-aminobutyric acid content gave recoveries of 97·5–101·8% (mean 99·6). Chromatograms of the γ-aminobutyric acid fraction from extracts of plant material, developed in three solvents, showed no other ninhydrin-reacting substance and the fraction did not form a complex with $CuCO_3$ (Crumpler & Dent, 1949).

Grass and silage

Grass A was a lawn-grass mixture containing no ryegrass. It was cut on 8 October 1956 and was 4–5 in. high; it had already been cut several times during the summer. Nitrogen content was 3·14% of dry matter. Grass B was mixed perennial and Italian ryegrass, first-year growth, cut on 28 May 1956, 12–18 in. high, not in flower. Nitrogen content was 3·34% of dry matter.

Silages from grasses A and B are laboratory silages prepared as described by Kemble (1956) without additions except to the metabisulphite silage, which consists of grass mixed intimately with dry sodium metabisulphite (0·2 g./50 g. of grass): the pH is higher than in ordinary silage, the metabisulphite acting as a bacteriostatic substance and the resulting product exhibits a very high degree of proteolysis with little ammonia formation (Macpherson, Wylam & Ramstad, 1957).

All results quoted in the tables are means of at least two determinations. The experiments with lawn and ryegrass have been selected as typical of numerous other ensilage and wilting experiments, all of which have shown similar trends in amino acid distribution.

RESULTS AND DISCUSSION

Formation of γ-aminobutyric acid in silage. The most striking feature of the results shown in Table 1 is the rapidity with which γ-aminobutyric acid is formed, and this is parallelled by a correspondingly rapid fall in the amount of free glutamic acid present. The initial increase in γ-aminobutyric acid is out of all proportion to the increase in the soluble nitrogen and it seems likely that much of it is produced from the free glutamic acid present, since this drops very sharply. Thereafter the rate of production falls off and the glutamic acid, enhanced by continuing proteolysis, increases again.

Formation of γ-aminobutyric acid during wilting. Wilting was carried out as described by Kemble & Macpherson (1954). We have distinguished arbitrarily between 'dry-wilting', where moisture is lost fairly rapidly, and 'moist-wilting', where the moisture content is sustained. Both processes are largely aerobic. It can be seen from Table 1 that in

Table 1. *Free γ-aminobutyric acid, dicarboxylic acids, amides and pyrrolidonecarboxylic acid in grass during conservation*

Results except those for dry matter, are expressed as percentage of total nitrogen of the original material. PCA, Pyrrolidonecarboxylic acid.

Extracted from	Dry matter (%)	Soluble N	γ-Amino N	Glutamic acid N	Glutamine N/2	PCA N	Aspartic acid N	Asparagine N/2
Old lawn grass A								
Fresh grass	19·3	9·8	0·20	1·00	0·20	0·15	0·30	0·20
Dry wilt, 72 hr.	58·5	31·9	0·45	0·38	1·60	0·38	0·28	2·55
Moist wilt, 72 hr.	19·3	41·7	0·14	0·94	1·90	0·46	0·52	7·15
Moist, 72 hr.; then dry, 72 hr.	74·6	44·3	0·36	0·48	2·10	0·43	1·24	6·45
Silage, 1 week	—	68·0	3·60	1·86	0·20	0·34	2·60	0·55
Silage, 5 weeks	—	69·5	3·40	1·84	0	0·71	2·80	0·60
Young perennial ryegrass B								
Fresh grass	22·7	9·1	0·10	1·01	0·34	0·07	0·35	0·19
Dry wilt, 24 hr.	71·4	11·7	0·76	0·44	0·30	Trace	0·30	0·34
Dry wilt, 48 hr.	76·4	11·8	0·73	0·43	0·30	0·12	0·30	0·36
Moist wilt, 24 hr.	23·3	18·5	0·11	1·57	0·80	0·16	0·64	0·64
Moist wilt, 48 hr.	23·6	28·0	0·05	1·58	1·46	0·28	0·61	1·91
Moist, 24 hr.; then dry, 24 hr.	73·1	21·8	0·63	0·55	0·70	0·06	0·46	0·95
Silage, 9 hr.	—	30·6	2·30	0·39	0·20	Trace	0·34	—
Silage, 24 hr.	—	65·1	2·83	1·63	0·34	0·13	0·97	1·75
Silage, 48 hr.	—	75·1	2·93	—	—	—	—	—
Silage, 72 hr.	—	77·5	2·94	—	—	—	—	—
Metabisulphite silage	—	85·7	2·97	3·00	0·16	0·54	1·75	1·78
Other samples analysed								
Mature field silage	—	67·2	3·85	1·75	0	0·68	2·90	0·38
Incubated microbe-free grass, 11 days	—	61·7	2·40	0·45	0·82	0·38	0·77	2·03

'moist-wilting' there is no formation of γ-aminobutyric acid; glutamine, however, is formed. In dry-wilting, on the other hand, there is considerable formation of γ-aminobutyric acid, but none of glutamine. In the dry wilt of grass A, where drying was slower, the formation of γ-aminobutyric acid was somewhat less and that of glutamine higher than in dry wilts of grass B. When the grass was first moist-wilted and then dried, γ-aminobutyric acid was formed during drying. In another experiment, a microbe-free grass was incubated under mercury seal for 11 days and gave considerable γ-aminobutyric acid and a little glutamine. The grass was shown to be free from bacterial contamination throughout the experiment, the pH did not drop and the conditions were largely those of an anaerobic moist-wilting.

From these experiments it appears that γ-aminobutyric acid can be formed under aerobic conditions as in dry-wilting or under virtually anaerobic conditions as in laboratory silages and in the incubated microbe-free grass, where glutamine is not normally produced. Glutamine formation appears to limit the formation of γ-aminobutyric acid. In moist-wilting, where glutamine formation may be considerable, γ-aminobutyric acid is either not formed or, if it is produced, is immediately converted into glutamine (cf. Steward, Bidwell & Yemm, 1956). Dry-wilting conditions, due to lack of proteolysis and subsequent deamination, are probably inimical to amide formation.

In the experiments with the combination of moist- and dry-wilting, the γ-aminobutyric acid seems to be derived from glutamic acid, not from the glutamine already formed, and may be a precursor of glutamine.

We have found only 0·1–0·2% of the total nitrogen of fresh grass to be in the form of γ-aminobutyric acid. Although very small amounts may be formed between the time of cutting and inactivation of the enzymes by boiling water, it seems probable that most of that found occurs as such in fresh grass. The values are lower than those estimated by Synge (1951), but in his analysis the grass sap was exposed to a lengthy dialysis and ionophoresis and it seems likely that much of it may have been formed during this period.

Amino dicarboxylic acid balance in conservation. We have attempted to correlate the glutamic and aspartic acid found with the amounts expected from the degree of proteolysis. Kemble & Macpherson (1954) showed that protein breakdown during conservation appeared to be uniform, i.e. the residual protein is of similar amino acid composition to the original, and that there is no preferential liberation of any of the acids. Given the amino acid composition of the plant protein therefore we can calculate how much of an amino acid

we should expect to find in the extracts, knowing the value in an extract of the fresh material and the percentage proteolysis. This, of course, is an over-simplification since some synthesis undoubtedly goes on even in the dying cells, but the net effect is a general proteolysis. Table 2 shows the contents of glutamic acid and aspartic acid of a few representative grass proteins: these samples have been obtained from various Laboratories and all were prepared from mature pre-flowering growth of pure strains by the general method of mild alkaline extraction and acid precipitation. The proteins showed almost identical amino dicarboxylic acid composition and the small differences indicated are probably not significant.

The amount of free glutamic acid present in the extracts falls far short of the expected amounts, but if we assume the closely related γ-aminobutyric acid, pyrrolidonecarboxylic acid and glutamine to have come directly or indirectly from glutamic acid residues, the balance, if bound glutamic acid is taken into account, is generally satisfactory in silage (Table 3). Bound glutamic acid is determined by hydrolysis of the extracts. More than the expected amount is found when much glutamine is present, as in the moist wilts, and it is probable that the glutamine is derived at least partly from some carbon skeleton other than that of glutamic acid.

In aerobic wilting, particularly under conditions of sustained moisture content, very large amounts of asparagine are formed, much more than can arise from aspartic residues of the protein and any such balance would be invalid. During anaerobic incubation, however, as in silage, there is little asparagine formation and under these circumstances we have examined the aspartic acid balance. In the examples shown in Table 4, except in the moist wilt, the aspartic acid found even after hydrolysis is less than the value calculated from the degree of proteolysis, particularly in the week-old silage. We have not found significant amounts of β-alanine, the decarboxylation product analogous to γ-aminobutyric acid from glutamic acid, but Kemble (1956) noted that, in the earlier stages of ensilage and in anaerobic incubation of microbe-free grass, excess of alanine was present: this may account for some of the missing aspartic acid.

Table 2. *Amino dicarboxylic acids in grass proteins*

Source	Total N (% of dry protein)*	Glutamic acid N (% of total N)	Aspartic acid N (% of total N)
Perennial ryegrass	12·8	7·64	6·02
Italian ryegrass	12·5	7·65	6·06
Cocksfoot	14·2	7·70	6·03
Timothy grass (1)	12·6	7·60	6·20
Timothy grass (2)	11·2	7·50	6·20

* Not corrected for ash.

Table 3. *Glutamic acid balance in grass conservation*

Results are expressed as percentage of total nitrogen of the material. PCA, Pyrrolidonecarboxylic acid.

Material extracted	N of free glutamic acid, glutamine, PCA and γ-aminobutyric acid (from Tables 1 and 2)	N of glutamic and γ-aminobutyric acid present after hydrolysis of extract	Glutamic acid N expected from proteolysis
Fresh grass A	1·55	1·50	—
Moist wilt, 72 hr.	3·44	4·20	4·05
Silage, 1 week	6·00	6·10	6·00
Silage, 5 weeks	5·95	6·30	6·10
Fresh grass B	1·52	1·50	—
Moist wilt, 24 hr.	2·64	2·68	2·20
Moist wilt, 48 hr.	3·37	3·85	2·96
Moist, 24 hr.; then dry, 24 hr.	1·94	2·94	2·49
Metabisulphite silage	6·67	7·23	7·38

Table 4. *Aspartic acid balance in grass conservation*

Results are expressed as percentage of total nitrogen of the material.

Material extracted	Aspartic acid N and asparagine N/2 free in extract (from Tables 1 and 2)	Aspartic acid N present after hydrolysis of extract	Aspartic acid N expected from proteolysis
Fresh grass A	0·50	0·48	—
Moist wilt, 72 hr.	7·67	8·00	2·41
Silage, 1 week	3·15	3·30	4·00
Silage, 5 weeks	3·40	3·90	4·10
Fresh grass B	0·54	0·58	—
Metabisulphite silage	3·53	4·68	5·10

Table 5. *Determination of the amides*

Results are expressed as percentage of total nitrogen of grass A. PCA, Pyrrolidonecarboxylic acid.

Extract from	Glutamine N/2	Asparagine N/2	Glutamic acid N	Aspartic acid N	PCA
Moist wilt, 72 hr.	1·90	7·15	0·94	0·52	0·46
Extract hydrolysed at pH 6·5	—	7·15	0·90	0·52	2·10
Extract hydrolysed with N-acid	—	—	3·10	7·62	1·10

Amides and pyrrolidonecarboxylic acid. The methods for the amides appear to be reliable when considerable amounts are present but the value for glutamine is less reliable when the amounts are small since it is affected by ammonia liberated from other substances in the extracts (Chibnall & Westall, 1932; Vickery *et al.* 1935). Even the pyrrolidonecarboxylic acid determination by itself does not provide a satisfactory check since a little of the glutamic acid also appears to be converted. The asparagine and aspartic acid remain unchanged during the hydrolysis of glutamine and asparagine is recovered quantitatively as aspartic acid on hydrolysis with N-acid (Table 5). In the hydrolysis with N-acid the same total glutamic acid + pyrrolidonecarboxylic acid is always obtained, but the final proportions of the two acids may vary. Most of the glutamine nitrogen appears in the pyrrolidonecarboxylic acid fraction on hydrolysis at pH 6·5, the conditions for glutamine determination; there is, however, a significant reduction in the glutamic acid present and this has been noted on several occasions. The aspartic acid nitrogen remains unchanged. Under the conditions for asparagine determination (hydrolysis by N-sulphuric acid at 100°) the asparagine appears as the additional aspartic acid; the considerable increase in total glutamic acid from this hydrolysis is discussed below.

The values obtained for pyrrolidonecarboxylic acid in grass and silage extracts are usually very small, but even so they will be over-estimates because of the partial conversion of glutamine during the preparation of the hot-water extracts. From the figures quoted by Vickery *et al.* (1935), we estimate this conversion to be about 20 % of the glutamine present under our conditions of extraction. In view of this and the proportions of pyrrolidonecarboxylic acid and glutamine found in grass (Table 1), we are of the opinion that pyrrolidonecarboxylic acid probably does not occur as such in the grasses examined. Figures for pyrrolidonecarboxylic acid in some of the silages are higher, particularly after some weeks, but there is simultaneous disappearance of glutamine and part of the pyrrolidonecarboxylic acid could arise from this; there may also have been some conversion of glutamic acid. The amount of pyrrolidonecarboxylic acid nitrogen in fresh grass B is 0·77 % of the soluble nitrogen, and the figure given by Ellfolk & Synge (1955) is estimated as 'much less than 0·86 %'.

Bound glutamic and aspartic acid. In general, fresh grass extracts showed little or no increase in the acids on hydrolysis. Under conditions of wilting, however, particularly where proteolysis is extensive, up to 1 % of the total nitrogen may be present as bound glutamic acid nitrogen, equivalent to about 5 % of the soluble nitrogen. Only slight increases were found in ordinary silage, but in grass preserved with metabisulphite 0·6 and 1 % respectively of the total nitrogen were present as bound glutamic and aspartic acid nitrogen (Tables 3 and 4).

The most interesting feature of these experiments is that all the bound glutamic acid found in wilted grass is liberated under the comparatively mild conditions used for the hydrolysis of asparagine, and no more is found on boiling with 6N-hydrochloric acid for 24 hr.; there may be slight destruction with the stronger acid in spite of the very large dilution of sample since N-acid usually gives significantly higher figures than 6N-acid. So far we have insufficient evidence to decide the structure of the bound glutamic acid, which may exist as glutamic, glutamine or pyrrolidonecarboxylic acid peptides (cf. Melville, 1935; Dekker, Stone & Fruton, 1949). A large part at least of the bound glutamic acid is, however, recoverable in the early fractions from the De-Acidite column and is not therefore acidic in nature.

SUMMARY

1. Simple quantitative methods, employing ion-exchange resins, are described for the determination of γ-amino-*n*-butyric, aspartic, glutamic and pyrrolidonecarboxylic acid in plant material.

2. The formation of the considerable amount of γ-aminobutyric acid found in silage and microbe-free grass is examined with respect to the glutamic acid and glutamine present. It seems to be derived from the glutamic acid and accumulates very rapidly under conditions unfavourable for the formation of glutamine.

3. Balances have been worked out comparing the glutamic and aspartic acid found with the amounts expected from the proteolysis involved. All the glutamic acid appears to be recoverable,

though a considerable part of it may exist as readily hydrolysable bound glutamic acid of as-yet undetermined structure.

4. The amounts of pyrrolidonecarboxylic and γ-aminobutyric acid found in extracts of fresh grass are very small, and pyrrolidonecarboxylic acid probably does not exist as such in the grass, the little found being formed during the extraction.

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Studies on Biosynthesis of Cholesterol

8. THE EFFECT OF Mg^{2+} AND Mn^{2+} IONS AND OF ADENOSINE TRIPHOSPHATE ON ENZYMIC ACTIVITY OF MICROSOMES IN STEROL BIOSYNTHESIS FROM MEVALONIC ACID*

By L. GOSSELIN† and G. POPJÁK
M.R.C. Experimental Radiopathology Research Unit, Hammersmith Hospital, London, W. 12

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It was shown in previous studies that for the synthesis of squalene and cholesterol from mevalonic acid by cell-free preparations of rat liver a fraction of soluble protein and microsomes were needed and that adenosine triphosphate and reduced pyridine nucleotides were essential cofactors in this enzyme system (Popják, Gosselin, Gore & Gould, 1958). It was further demonstrated that during aerobic conditions, when mostly cholesterol was synthesized, a reducing agent, such as glutathione or ascorbic acid, was needed to activate the system.

The soluble enzymes could be preserved very satisfactorily, after precipitation with ammonium sulphate at $-15°$, but the microsomes progressively lost their activity when kept frozen at $-15°$ or at $-79°$. Consequently in our previous work microsomes were prepared freshly for each experiment.

The present paper describes a study of the 'ageing' process of the microsomes and of the conditions leading to the restoration of the ability of old preparations of microsomes to catalyse the synthesis of sterol from mevalonic acid in the presence of dialysed soluble enzymes and of the appropriate coenzymes. It was possible to reactivate fully preparations of microsomes that were kept frozen at $-15°$ for 4 months and which had lost, apparently, 90% of their activity. A brief account of the results has already been presented (Gosselin & Popják, 1958).

METHODS

The methods and materials used in the present investigation were essentially those previously described (Popják *et al.* 1958).

Testing of enzymic activity of microsomes. The criterion of enzymic activity of the microsomes was their ability to catalyse the synthesis of digitonin-precipitable sterols from [2-^{14}C]mevalonate when combined with dialysed soluble liver enzymes in the presence of adenosine triphosphate

* Part 7: Cornforth, Cornforth, Pelter, Horning & Popják (1959).

† Aspirant du Fonds National de la Recherche Scientifique (Belgium).

(ATP), pyridine nucleotides, nicotinamide, Mg^{2+}, ascorbate and oxygen. Since for the synthesis of sterols soluble enzymes as well as the cell particles are needed, the activity of the aged preparations of microsomes was usually assayed against an enzyme system which contained microsomes prepared on the day of the experiment or a few days previously, the same preparation of the soluble enzymes being employed in the control and test systems.

The dialysed soluble enzymes and microsomes were prepared as described previously (Popják *et al.* 1958) from homogenates made according to Bucher & McGarrahan (1956).

When a preparation of microsomes is described as being 4 days old or 4 months old, it means that the suspension of microsomes (in 0·1 M-KH_2PO_4–K_2HPO_4 buffer, pH 7·5, containing 0·03 M-nicotinamide and 4 mM-$MgCl_2$) was kept frozen at $-15°$ for that length of time after the day of its preparation.

The suspensions of microsomes were so adjusted that 0·1 ml. contained an amount of microsomes equal to that present in 1 ml. of the liver homogenate from which they had been prepared.

Incubations. All incubations were made in conical flasks at 37° for 1 hr. with air as gas phase in the flasks. In most experiments a basic 'standard incubation mixture' was employed consisting of: dialysed soluble enzymes (1 ml., 30–50 mg. of protein); suspension of microsomes (0·2 ml.); reduced diphosphopyridine nucleotide (DPNH; 1 μmole); triphosphopyridine nucleotide (TPN; 1 μmole); ATP (10 μmoles); [2-^{14}C]mevalonate (1 μmole); potassium ascorbate (30 μmoles); KH_2PO_4–K_2HPO_4 buffer, 0·1 M, pH 7·5; nicotinamide, 0·03 M; $MgCl_2$, 4 mM; final volume, 4 ml. Additions to, and deviations from, this standard mixture are indicated in the tables. The concentrations of the reagents in the 'standard incubation mixture' were found previously to be optimum for a liver-enzyme system in which freshly prepared microsomes were employed. Extraction of cholesterol, preparation of digitonides and assay of ^{14}C were all done as described by Popják *et al.* (1958). Since the yields of sterol digitonides were nearly identical in all incubations (about 7 mg.) and since the 'infinite thickness' count is proportional to the specific activity (1600 counts/min./2 cm.^{2} at infinite thickness is equivalent to 10^{-3} μc/mg. under our counting conditions), all results are expressed in terms of the infinite-thickness counts.

Artificial ageing of microsomes. This was done by incubating in a test tube the usual concentrated suspension of microsomes (prepared on the day of the experiments) at 37° for a period ranging from 15 to 60 min. At the end of the required period the preparations were cooled in ice and kept at ice temperature until the test incubations were set up.

DL-[2-^{14}C]*Mevalonic acid.* This substance synthesized according to Cornforth, Cornforth, Popják & Gore (1958), was obtained from the Radiochemical Centre, Amersham, Bucks. Material with a specific activity of 0·2 μc/μmole was used.

RESULTS

When microsomes stored for 1 month at $-15°$, or at $-79°$, were tested in combination with freshly prepared and undialysed soluble enzymes [supernatant of homogenates centrifuged at 105 000 *g* for 1 hr. (S_{105})], the particles appeared to have lost approximately one-half of their activity. On the other hand, when these same particles were tried in a reconstituted system, containing dialysed soluble enzymes and the recognized cofactors (Popják *et al.* 1958), they were unable to catalyse efficiently the synthesis of cholesterol from mevalonic acid. This observation suggested at first that the fresh S_{105} preparations and microsomes contained some common, as yet unidentified, factor, which was lost from microsomes during storage and was removed from the soluble enzymes by precipitation with ammonium sulphate and by dialysis.

Activation of microsomes stored at $-15°$ by Mn^{2+} and Mg^{2+} ions and by adenosine triphosphate

Several unsuccessful attempts were made to preserve the activity of the stored microsomes by the addition of glutathione, cysteine or ascorbic acid to their suspensions before freezing or by the addition of coenzyme A and of vitamin B_{12} to the incubation mixtures. It was found, however, that preparations of microsomes, kept frozen at $-15°$ for as long as 4 months, could be largely reactivated by Mn^{2+} or by an increased concentration of Mg^{2+} ions in the incubation mixture.

The results of the two experiments shown in Table 1 illustrate the strong 'rejuvenating' effect of mM-Mn^{2+}, in the presence of 4 mM-Mg^{2+} ions, on microsomes inactivated by storage at $-15°$ for 7 and 11 weeks respectively.

Apart from manganese, increasing amounts of ATP and addition of sodium fluoride also had a reactivating effect on stored microsomes; this was further enhanced by the presence of Mn^{2+} (mM) (Table 2).

So far the results suggested that storage of microsomes at low temperatures caused both a loss of bivalent cations from the microsomes, leading to an inactivation of the enzymes, and possibly also a liberation of adenosine triphosphatase, which destroyed one of the essential coenzymes of the

Table 1. *Reactivation of stored microsomes by Mn^{2+} ions*

Each flask contained the standard incubation mixture (see Methods), but with 0·5 μmole of TPN. Either freshly prepared or old microsomes were used as shown. The old microsomes employed in Expt. 1 were stored at $-15°$ for 7 weeks and those in Expt. 2 for 11 weeks.

Microsomes used and additions	Specific activity of sterol digitonides (counts/min. at infinite thickness)	
	Expt. 1	Expt. 2
Fresh, none	1570	7438
Old, none	361	957
Old + mM-Mn^{2+}	1159	5004

Table 2. *Reactivation of aged microsomes by adenosine triphosphate, Mn^{2+} ions and by sodium fluoride*

Each flask contained the standard incubation mixture but with 15 μmoles of ATP and 2 μmoles of DPNH. Additions to this mixture were as shown.

Age of microsomes	Additions (μmoles)	Specific activity of sterol digitonides (counts/min. at infinite thickness)
4 days	None	2177
	Mn^{2+}, 4	4058
	ATP, 15	5157
	ATP, 15 + Mn^{2+}, 4	6832
4 months	None	231
	Mn^{2+}, 4	1259
	ATP, 15	2347
	ATP, 15 + Mn^{2+}, 4	3369
	NaF, 40	606
	ATP, 15 + Mn^{2+}, 4 + NaF, 40	5840

Table 3. *Cation requirements in sterol synthesis and ageing of microsomes*

Each flask contained: soluble enzyme, 1 ml.; suspension of microsomes stored previously at −15°, 0·2 ml.; DPNH, 2 μmoles; TPN, 1 μmole; ATP, 30 μmoles; potassium ascorbate, 30 μmoles; [2-^{14}C]mevalonate, 1 μmole. The final volume (4 ml.) contained KCl (0·15 M) buffered to pH 7·5 with 2-amino-2-hydroxymethylpropane-1:3-diol/HCl (0·01 M) and nicotinamide (0·03 M). $MgCl_2$ and $MnCl_2$ were added as indicated. In Expt. *A* the microsomes used were 9 days and in Expt. *B* 11 weeks old.

Cation addition (μmoles)	Specific activity of sterol digitonides (counts/min. at infinite thickness)	
	Expt. *A*	Expt. *B*
Mg^{2+}, 16	3977	2551
Mg^{2+}, 20	9810	3023
Mg^{2+}, 16 + Mn^{2+}, 4	9041	3620
Mn^{2+}, 20	6710	2039

Table 4. *Cation requirement with fresh microsomes; comparison of Mn^{2+} with Mg^{2+} ions*

Each flask contained: soluble enzyme, 1 ml.; microsome suspension prepared on the same day in magnesium-free medium (0·1 M-potassium phosphate buffer, pH 7·5, + nicotinamide 0·03 M), 0·2 ml.; DPNH, 1 μmole; TPN, 0·5 μmole; ATP, 20 μmoles; ascorbate, 30 μmoles; [2-^{14}C]-mevalonate, 1 μmole. The final volume (4 ml.) contained 0·1 M-potassium phosphate buffer (pH 7·5) and nicotinamide (0·03 M). $MgCl_2$ and $MnCl_2$ were added as indicated.

Additions (μmoles)	Specific activity of sterol digitonides (counts/min. at infinite thickness)
None	0
Mg^{2+}, 16	8317
Mn^{2+}, 16	4270
Mg^{2+}, 16 + Mn^{2+}, 4	6178
Mg^{2+}, 20	7261
Mn^{2+}, 20	4655
Mg^{2+}, 8 + Mn^{2+}, 8	8167

system. This view is supported by the fact that the highest activity resulted from incubating a mixture containing a high concentration of ATP, Mn^{2+} and of sodium fluoride.

In further experiments it was found that the effect of Mn^{2+} ions was not specific, as reactivation could be achieved with equivalent amounts of Mg^{2+} ions. This was shown by experiments presented in Table 3, in which a system partially reactivated by an increased concentration of ATP was used.

The need for the bivalent cations may be shown also in systems containing microsomes prepared on the day of the experiment: this need is absolute, as in the absence of either Mg^{2+} or Mn^{2+} ions the liver enzyme system fails to convert mevalonic acid into cholesterol (Table 4). However, in the presence of fresh microsomes a final concentration of 4 mM of Mg^{2+} or 2 mM-Mg^{2+} + 2 mM-Mn^{2+} appears to be the optimum, but Mn^{2+} cannot entirely replace Mg^{2+}. The need for a higher concentration (5 mM) of bivalent cation became apparent only when aged preparations of microsomes were used, as was verified in several experiments; with fresh preparations of microsomes an increased concentration of Mg^{2+} (5 mM) or the addition of Mn^{2+} (mM) to 4 mM-Mg^{2+} ions did not enhance the synthetic activity of the enzyme system; on the contrary slight inhibiting effects were observed occasionally under such conditions.

Artificial ageing of microsomes

The inactivation of microsomes, which manifests itself in a need for Mn^{2+} in addition to Mg^{2+} ions, or for a greater concentration of Mg^{2+} and of ATP, proceeds slowly and variably at −15°, but it can

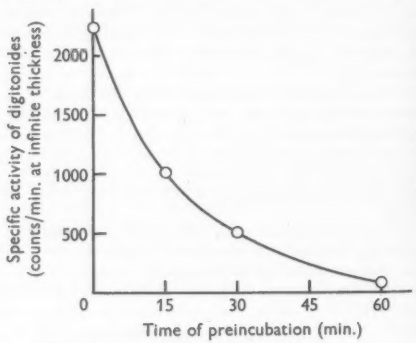

Fig. 1. Rapid ageing of microsomes at 37°. Each point is the result of an incubation carried out under standard conditions, except that the microsomes, prepared on the day of the experiment, were preincubated at 37° for 15–60 min. and then cooled in ice before use.

be much accelerated by incubation of microsomes at 37°. In the experiment recorded in Fig. 1 the microsomes used were prepared on the day of the experiment and their suspension (in 0·1 M-phosphate buffer containing 0·03 M-nicotinamide and 4 mM-$MgCl_2$) was incubated at 37° for 15–60 min. before use. If the ability of the microsomes, in the presence of soluble liver enzymes, to catalyse the synthesis of sterols from mevalonic acid in our standard incubation mixture is taken as a measure of their activity, it can be seen from Fig. 1 that the cell particles were inactivated rapidly and at a steady rate by previous incubation at 37°. The curve in Fig. 1 can be represented by a single exponential equation with a half-time of about 15 min. The inactivation of the microsomes at 37° is, apparently, equivalent to that induced by storage at low temperatures, as it can be fully reversed by increased concentrations of ATP and of Mg^{2+} ions [cf. Table 6, incubations (*a*)–(*d*)].

Relationship between concentrations of Mg^{2+} ions, adenosine triphosphate and of microsomes: appearance of inhibitors of sterol synthesis in aged preparations of microsomes

The main limiting factor in the observed activity of aged preparations of microsomes is the concentration of ATP used in the incubations. In previous studies (Popják *et al.* 1958) the optimum concentration of ATP for sterol synthesis from mevalonic acid was 2·5 mM (10 μmoles of ATP in 4 ml. incubation) when freshly prepared microsomes and 0·5–2·0 μmoles of DL-mevalonate were employed in the enzyme system; higher concentrations of ATP were often inhibitory. These results were confirmed in the present investigation, and it was found that there existed a close correlation between the amounts of ATP, Mg^{2+} and of microsomes in the incubations and the observed synthetic activity (Table 5). When fresh microsomes were used the

Table 5. *Magnesium, adenosine triphosphate and microsome interrelationship*

Each flask contained: soluble enzyme, 1 ml.; DPNH, 2 μmoles; TPN, 1 μmole; ascorbate, 30 μmoles; [2-^{14}C]mevalonic acid, 1 μmole; the amounts of $MgCl_2$, ATP and of microsome suspensions used were as indicated. The final volume (4 ml.) contained 0·1 M-phosphate buffer (pH 7·5) and nicotinamide (0·03 M).

Microsome preparation	Vol. of microsome suspension (ml.)	Specific activity of sterol digitonides (counts/min. at infinite thickness)			
		Mg 16 μmoles ATP 10 μmoles	Mg 16 μmoles ATP 30 μmoles	Mg 20 μmoles ATP 10 μmoles	Mg 20 μmoles ATP 30 μmoles
Fresh*	0·4	5749	—	—	—
	0·2	5637	7453	4799	6089
	0·1	6039	2881	4968	5435
Aged†	0·4	613	—	—	—
	0·2	1569	4648	1968	5352
	0·1	3980	5147	2878	5006

* Microsomes prepared on the day of the experiment.
† Fresh microsomes preincubated at 37° for 30 min., then cooled in ice before use.

Table 6. *Reactivation of microsomes aged at 37° and the presence of inhibitors of sterol synthesis in aged preparations*

Each flask contained the standard incubation mixture except for the variations of the volume of microsome suspensions. The nature of the microsomes and additions was as shown. The reactivating factors [incubations (*b*), (*d*) and (*h*)] were: ATP, 20 μmoles; Mg^{2+}, 4 μmoles and NaF, 40 μmoles. The fresh microsomes were prepared on the day of the experiment; the 'aged' microsomes used in incubations (*c*), (*d*) and (*f*) were fresh microsomes preincubated at 37° for 40 min. and then cooled down in ice before use. In incubations (*g*), (*h*) and (*i*) 'Sp' and 'Sd' represent the supernatant and sediment respectively of a suspension of microsomes kept at −15° for a few months and centrifuged at 105 000 *g* for 60 min. 'Sd' was resuspended in the usual amount of fresh buffer.

Nature of microsomes and additions	Specific activity of sterol digitonides (counts/min. at infinite thickness)
(*a*) Fresh microsomes (0·2 ml.)	4247
(*b*) Same as (*a*) + re-activating factors	7532
(*c*) Aged microsomes (0·2 ml.)	642
(*d*) Same as (*c*) + re-activating factors	7572
(*e*) Fresh microsomes (0·1 ml.)	2860
(*f*) Fresh and aged microsomes (0·1 ml. of each)	1228
(*g*) Fresh microsomes (0·2 ml.) + 'Sp' (0·2 ml.)	1958
(*h*) Same as (*g*) + re-activating factors	6041
(*i*) Fresh microsomes (0·1 ml.) + 'Sd' (0·1 ml.)	264

concentrations of these three components of the enzyme system were not very critical, except that a high concentration of ATP (30 μmoles/4 ml.) was inhibitory when the amount of microsomes was reduced to 0·1 ml. This inhibitory effect was largely counteracted by an increased concentration of Mg^{2+} ions (Table 5; experiment with fresh microsomes). After ageing of the particles by incubation at 37° this inhibitory effect of ATP disappeared and the high requirement for ATP became manifest.

The results of the experiment with the preincubated microsomes, shown in Table 5, also suggest that during ageing some inhibitory substance(s) might be released from the microsomes since, for a given level of ATP and Mg^{2+} concentration, an increase in the microsome content resulted in a proportionate lowering of the observed synthesis of sterol. It is shown in Table 6 that the addition of aged microsomes, or of a particle-free supernatant of old microsomes, to an enzyme system containing fresh microsomes resulted in a serious impairment of sterol synthesis. The inhibition caused by the supernatant of old microsomes was abolished by high concentrations of ATP, magnesium and sodium fluoride.

DISCUSSION

The main point which arises from the present observations is that ageing of the microsome suspensions (whether induced slowly at −15° or rapidly by incubation at 37°) causes an increased requirement for ATP and for Mg^{2+} (or Mn^{2+}) ions by the liver-enzyme system synthesizing sterol from mevalonic acid. Our observations on the reactivation of old microsomes by mM-Mn^{2+} ions in the presence of 4 mM-Mg^{2+} ions suggested at first that the loss of enzyme activity was associated with the loss of cations from the particles. However, the need for more ATP and the appearance of some inhibitory substance(s) after ageing of microsomes showed that the inactivation process was more complex. More ATP may be needed by the liver-enzyme system after ageing of the microsomes because of a loss of ATP through an increased adenosine triphosphatase activity. This idea is supported by the fact that sodium fluoride, which by itself had no definite power to reactivate old microsomes, enhanced the synthetic activity of the enzyme system in the presence of high concentrations of ATP. However, the effect of sodium fluoride could be observed only with very old preparations of microsomes, whereas the need for more ATP and Mg^{2+} (or Mn^{2+}) ions appeared within a few days of storage at −15°. With microsome preparations at least up to 8 days old sodium fluoride had no effect, even in the presence of high concentrations of ATP (Table 7). This suggests an additional mechanism for loss of ATP, possibly through chelation with some substance (e.g. Ca^{2+} ions) released from the microsomes. Ca^{2+} ions in a concentration of mM inhibited sterol synthesis by 75 %, but this was only partly counteracted by an increased concentration of Mg^{2+} ions. It is, therefore, difficult to decide whether the activating effect of Mg^{2+} or Mn^{2+} ions on microsomes may be attributed solely to a replacement of metal ions lost, or to a neutralizing of inhibitors released from the particles during ageing; both mechanisms may operate. The results shown in Table 6 indicate that the inhibitory effect of the supernatant of an aged microsomal preparation was counteracted by the reactivating system.

A practical consequence of the present study is that suspensions of microsomes kept frozen at −15° for at least as long as 4 months may be used satisfactorily in a cholesterol-synthesizing enzyme system provided that the concentration of Mg^{2+} ions is raised to 5 mM and that of ATP to 7·5 mM (cf. 'standard incubation mixture' under Methods); by the inclusion of sodium fluoride (10 mM) the synthetic activity is raised further so that the results obtained cannot be distinguished from those obtained with a system in which fresh microsomes are employed.

Table 7. *Effects of adenosine triphosphate and of sodium fluoride on enzyme system containing microsomes* 8 *days old*

Each flask contained the standard incubation mixture with microsomes stored at −15° for 8 days. Additions were as shown.

Additions (μmoles)	Specific activity of sterol digitonides (counts/min. at infinite thickness)
None	5 156
ATP (20)	13 997
NaF (40)	5 238
ATP (20) + NaF (40)	12 324

SUMMARY

1. The enzyme system from rat liver, consisting of a soluble protein fraction and microsomes, and which synthesizes cholesterol from mevalonic acid, was investigated for stability of the microsomal enzymes.

2. Microsomes kept frozen at −15° for a few weeks to a few months are unable to catalyse sterol synthesis when combined with the soluble enzymes under conditions optimum for an enzyme system containing fresh microsomes.

3. Inactivation of fresh microsomes can be induced rapidly by incubation at 37°.

4. Partial reactivation of microsomes stored at −15°, or of those 'aged' at 37°, can be achieved by

the addition of Mn^{2+} ions (mM) or by an increased concentration of Mg^{2+} ions (5 mM). The sterol-synthesizing system has an absolute requirement for Mg^{2+} or Mn^{2+} ions.

5. Enzyme systems containing old preparations of microsomes have also a requirement for adenosine triphosphate higher than those containing fresh microsomes. Sodium fluoride, which has no effect on fresh preparations, enhances the stimulating effects of high concentrations of adenosine triphosphate on old preparations of microsomes.

6. During ageing, substances inhibiting sterol synthesis appear in the microsomal preparations.

7. Microsome preparations as old as four months can be fully reactivated by a suitable combination of Mg^{2+} ions, adenosine triphosphate and sodium fluoride.

We wish to thank Mr Terry Flynn for his skilful technical assistance.

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The Metabolism of [16-^{14}C]Oestrone *in vitro*

By P. H. JELLINCK*
Department of Biochemistry, McGill University, Montreal, Canada and the Medical College of St Bartholomew's Hospital, London, E.C. 1

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Since the original demonstration by Zondek (1934) that liver mince rapidly inactivates oestrogens, many workers have studied the metabolism of oestrone and oestradiol *in vitro*, employing loss of biological activity to follow hormone-degradation (Heller, 1940; Engel & Rosenberg, 1945; Levy, 1947; De Meio, Rakoff, Cantarow & Paschkis, 1948; Pearlman & De Meio, 1949). Ryan & Engel (1953), by means of countercurrent distribution and fluorometric analysis, found that after incubation of these two natural oestrogens with rat-liver slices over 50% of the starting material was converted into unknown metabolites. More recent experiments with [^{14}C]oestrogens *in vivo* have supported these findings. Thus Valcourt, Thayer, Doisy, Elliott & Doisy (1955), on administering [16-^{14}C]oestrone to rats, found that even after enzymic hydrolysis of conjugates only one-third of the ^{14}C excreted in bile and urine was extractable with ether, a solvent in which steroid oestrogens are readily soluble. Similarly, Beer & Gallagher (1955) showed that extraction with ether failed to remove all the radioactivity from hydrolysed urine of human patients injected with [^{14}C]oestrone or [^{14}C]oestradiol, and, in perfusion experiments with radioactive oestrone, Wotiz, Ziskind & Ringler (1958) observed a large amount of chloroform-insoluble material containing ^{14}C in the hydrolysed dialysates of rat plasma.

* Present address: Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1.

Very little is known about the nature of the inactivation products of oestrogens, but there is evidence that oestrone and oestradiol are attacked by an oxidative enzyme system. Thus Heller (1940) found that cyanide inhibited the inactivation of oestrogens by liver, suggesting that an oxidase was involved, and in support of this theory Westerfeld (1940) and Graubard & Pincus (1941) showed that oxidases from plant sources were capable of acting on oestrogens. Levy (1947), using rat-liver homogenates, also showed inhibition by cyanide as well as by azide and carbon monoxide, and suggested that the cytochrome system was involved. He found that inactivation of oestradiol was greatly reduced under anaerobic conditions, and this was confirmed by De Meio *et al.* (1948), who, however, found very little inhibition of oestrogen-inactivation by cyanide or azide and none by malonate, iodoacetate or fluoride. In all these experiments very little conjugation was observed and the results which were obtained by bioassay methods give no indication about the extent to which the original steroid molecule had been altered.

The advent of isotope-labelled oestrogens, however, made the solution of this problem more feasible, and the present investigation was therefore undertaken to determine whether the water-soluble ether-insoluble metabolites observed *in vivo* are also formed *in vitro* and, if so, to try and discover the nature of these products and the effect of enzyme inhibitors on their formation.

EXPERIMENTAL

Materials. [16-^{14}C]Oestrone prepared by Thompson (1951) was shown to be radiochemically pure by chromatography with the toluene–propylene glycol system (Jellinck, 1953) followed by radioautography of the developed chromatogram. For this purpose, enough radioactive material was used to enable 0·1% of impurity to be detected.

Ether was shaken with an acidified solution of $FeSO_4$ to remove peroxides, and distilled; ethanol was distilled over CaO. Toluene (thiophen-free) and methylene chloride used for chromatography were both distilled.

Buffer solutions. Phosphate–saline (Krebs & Eggleston, 1940), pH 7·4, was used for the liver, and Krebs–Ringer bicarbonate solution (Cohen, 1949) for the placental incubations. The medium was 25 mM-sodium phosphate buffer, pH 6·1, for the mushroom tyrosinase experiment and no buffer was added to the blood. The pH of all buffer solutions was measured with a standardized glass electrode before use and the pH of each mixture was checked with indicator at the end of the experiment.

Tissues. Rat liver was obtained from young adult male animals killed by a blow on the back of the neck, and the slices were prepared with a Stadie–Riggs tissue slicer. To prepare the homogenate, rat liver in phosphate saline was disintegrated for 5 min. in a tissue blender (MSE Ato-Mix). A small piece of human liver with no visible signs of abnormality was obtained after laparotomy from a 56-year-old female patient.

The human placentae (full-term) were placed immediately after delivery into ice-cold NaCl solution (0·154 M) and small pieces were removed with scissors from both the maternal and foetal sides. They were then ground with coarse sand in a mortar under Krebs–Ringer bicarbonate solution.

Human whole blood which had been stored for 24 hr. at 4° with added sodium citrate (25 mM) and glucose (0·4%) was used.

Mushroom tyrosinase. Fresh mushrooms (5 g.) were ground with sand under water (25 ml.) and centrifuged after straining through muslin. The activity of the preparation was tested with catechol as substrate by the method of Wills & Wormall (1950). This crude extract would, of course, contain several phenol oxidases.

Assay of radioactivity. This was carried out with a windowless gas-flow counter (Nuclear Instruments, Chicago, Ill., U.S.A.) operating at 40–50% efficiency or with a thin mica-window Geiger–Müller tube (Ecko type EHM 2S) coupled to a scaler and E.H.T. unit (Panax type D 554). The samples for plating, with the exception of the aqueous ones, were dissolved in ethanol and evaporated at 60–70° under an infrared lamp. Copper disks (diameter 2·5 cm.) were used for the windowless counter and stainless-steel cupped planchets (internal diameter 1·5 cm.) for the end-window instrument and for the $BaCO_3$ assay. Whenever possible, samples were plated at infinite thinness (100 μg. or less of solids) or the count was corrected for self-absorption after the addition of a steroid standard of very high specific activity and negligible weight (Heard *et al.* 1954). Carbon dioxide collected during the incubation of rat liver was precipitated as $BaCO_3$ by a procedure described by Calvin (1949). Half the total quantity of solid was transferred as a slurry in ethanol to a stainless-steel cupped planchet, dried in an oven at 110–120° and assayed with the windowless counter. Tissues were dried, pulverized in a mortar and counted in the same way as the $BaCO_3$. Samples were plated in duplicate and counting was continued long enough to give a statistical accuracy of 5% or better. A standard count with [4-^{14}C]testosterone was taken daily and the counts given by the samples were corrected for background.

Chromatography. The methods were as described by Burton, Zaffaroni & Keutmann (1951) and Axelrod (1953), except for slight modifications. Strips of Whatman no. 1 filter paper (1 cm. × 34 cm.), which had been washed successively with acetic acid (2 N), water, methanol and toluene, were dipped into the stationary phase (formamide–methanol, 1:1, or propylene glycol) and well blotted between filter papers. The mixtures under investigation were dissolved in ethanol (0·1–0·2 ml.) for application to the paper and the spots dried in a stream of N_2 to prevent the formation of any steroid-oxidation products. The chromatograms were developed by downward flow of toluene or methylene chloride which had been equilibrated with the stationary phase. The tanks were maintained at a constant temperature of 25°. In both systems used the solvent was allowed to run off the end of the paper strip so that the rates of movement of metabolites are not expressed as R_F but relative to that of a faster-moving reference compound.

Radioautography. The dried chromatograms were stapled on to X-ray film (Kodak No Screen) and kept in close contact inside cassettes for a period depending on the radioactivity of the material. For ^{14}C, 24 hr. was found adequate for activities greater than 0·005 μc/cm.^{2} (10^4 disintegrations/min./cm.^{2} approx.).

Incubations. The liver slices (0·8–1·0 g. wet wt.) and medium (25 ml.) which had been saturated with O_2 were shaken for 3 hr. at 38° in conical flasks (100 ml.) with tight-fitting rubber stoppers. The radioactive oestrone was added dissolved in ethanol (0·1 ml.) and the CO_2 evolved during the incubation was absorbed in 30% KOH (2 ml.) contained in small test tubes hanging vertically from the stoppers. The liver homogenate (0·8–1·0 g. wet wt.) in phosphate–saline (25 ml.) was treated in a similar way but no CO_2 was collected.

The ground placental tissue (8–9 g. wet wt.) in Krebs–Ringer bicarbonate solution (60 ml.) was shaken for 3 hr. at 38° in conical flasks (250 ml.) with $CO_2 + O_2$ (5:95) bubbling through the liquid at a slow rate during the entire incubation period. Glucose (0·1 g.) was also added to the medium and the oestrone was dissolved in 0·5 ml. of propylene glycol.

Blood (30 ml.) was incubated with shaking in a conical flask (100 ml.) at 38° for 2 hr., during which O_2 was bubbled through continuously. The added oestrone was dissolved in 0·3 ml. of propylene glycol.

The buffered mushroom extract (25 ml.) was incubated for 3 hr.

Extraction and fractionation. The procedure (Fig. 1) was identical for the liver slices and the placental brei. The medium was decanted immediately after incubation, filtered and the tissues were washed twice with 0·6 vol. of water. The liver homogenate was centrifuged before decantation. The medium and aqueous washings and also the mushroom tyrosinase solution were extracted three times with equal volumes of ether and the tissues were kept

overnight under ethanol (20 ml.), filtered and washed twice more with this volume of solvent. The ethereal fraction was dried over anhydrous Na_2SO_4, filtered and the solvent removed *in vacuo* at 25°. The residue from the ethereal fraction was dissolved in ethanol for assay of radioactivity and the ethanolic and aqueous fractions were plated without further treatment.

The blood was centrifuged and the cells were washed four times with an equal volume of KCl (0·154 M). The plasma was acidified with HCl (0·01 N) to pH 6·5 (glass electrode) and extracted with three equal volumes of ether. The extract was dried, filtered, evaporated to dryness and dissolved in ethanol for assay of ^{14}C. The erythrocytes were haemolysed by the addition of an equal volume of ethanol–ether (3:1, v/v), well stirred, centrifuged and washed three times with an equal volume of ethanol.

Examination of aqueous fractions. Portions (5 ml.) of the aqueous solutions were acidified to pH 1–2 with 12 N-HCl and extracted with an equal volume of ether or butan-1-ol. The pH was adjusted to 12–13 with N-NaOH and hydrolysis was carried out by refluxing with 7% (w/v) HCl for 3 hr. In all cases the amount of ^{14}C removed by ether or butanol from the aqueous fractions was determined from the difference between the activities of the aqueous fractions before and after extraction.

Examination of ethereal fractions. Portions of the dried ethereal fractions, usually containing over 0·005 μc of ^{14}C, were examined for oestrone and oestradiol with the toluene–propylene glycol system followed by radioautography. Compounds with R_F values much smaller than that of oestradiol were eluted from the appropriate sections of the paper strip with methanol (3 × 5 ml.) and after concentration of the eluate by evaporation in a stream of N_2 they were re-examined by chromatography on the methylene chloride–formamide system and radioautography. The eluted compounds were also analysed by the isotope-dilution technique (Francis, Mulligan & Wormall, 1954). The added oestriol (18 mg.) was crystallized from ethanol.

RESULTS AND DISCUSSION

The results in Table 1 show that liver and the phenol oxidases from mushrooms have a marked ability for converting oestrone into water-soluble ether-insoluble metabolites and that blood and placental breis are ineffective in this respect.

A considerable proportion of the ^{14}C cannot be removed from fresh or boiled liver slices by washing with water and this adsorption is also observed with placental tissue and to a smaller extent with blood.

The formation of water-soluble, ether-insoluble radioactive products by rat-liver slices is almost completely abolished by cyanide, but malonate had no inhibitory effect.

The persistence of a small but significant amount of radioactivity in the liver even after repeated extraction with ethanol may represent actual incorporation of ^{14}C into the tissue, but this will need further verification. In the presence of cyanide the rat-liver slices retained only negligible amounts of radioactivity, in spite of the fact that there was more adsorbed ^{14}C under these conditions, as shown by the activity of the ethanolic extracts. Once again malonate had no effect on this 'incorporation'.

The discrepancies in total recovery of radioactivity are most marked when small weights of [^{14}C]oestrone are used and are due to losses in manipulation, probably by adsorption on to the glassware. Thus losses were observed even in the absence of tissue, and with boiled liver. The greatest discrepancies, however, occurred whenever water-soluble metabolites were formed, and since no radioactive carbon dioxide was evolved during the incubations, it may be that some of the oestrone-breakdown products are volatile and are lost when the samples are evaporated to dryness for counting. Another source of error arises in assaying the dry tissue powder for ^{14}C, since the count rates cannot be corrected reliably for self-absorption. One more minor (< 5%) contributing factor is the counting error.

Another object of the experiment was to gain information about the nature of the ether-insoluble

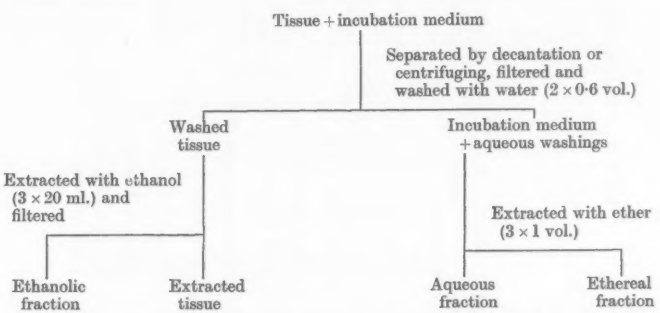
Tissue + incubation medium
Separated by decantation or centrifuging, filtered and washed with water (2 × 0·6 vol.)
Washed tissue
Incubation medium + aqueous washings
Extracted with ethanol (3 × 20 ml.) and filtered
Extracted with ether (3 × 1 vol.)
Ethanolic fraction
Extracted tissue
Aqueous fraction
Ethereal fraction

Fig. 1. Fractionation scheme: after incubation of [16-^{14}C]oestrone with tissues.

Table 1. *Percentage distribution of radioactivity in different fractions after incubation of* [16-^{14}C]*oestrone with tissues*

—, Not estimated.

Expt. no.	Tissue	Inhibitor (0·01 M)	Wt. of added oestrone (μg.)	Activity of added oestrone (10^{-3} counts/min.)	Percentage of added radioactivity: Aqueous medium after extraction with ether	Ethereal extract	Ethanolic extract of of tissue	Tissue after extraction with ethanol	CO_2 (as $BaCO_3$)	Total recovery	Total recovery corrected for control
1	Liver (rat)	—	160	126	30·3	9·1	23·6	3·6	<0·1	66·5	82·4
	Nil	—	160	126	1·7	79·0	—	—	<0·1	80·7	100
	Liver (rat)	NaCN	160	126	4·6	20·7	50·1	0·1	<0·1	74·0	91·7
2	Liver (rat)	—	100	5·4	35·2	9·6	10·4	1·5	—	56·7	65·3
	Liver (rat)	—	400	21·6	41·9	8·0	13·7	3·2	—	66·8	77·0
	*Liver (rat)	—	100	5·4	17·4	40·0	17·5	1·5	—	76·4	88·0
	Nil	—	100	5·4	0·6	86·2	—	—	—	86·8	100
	Boiled liver (rat)	—	100	5·4	2·2	25·3	50·9	0·5	—	78·9	90·9
	Liver (rat)	NaCN	100	5·4	1·9	39·5	37·7	0·3	—	79·4	91·5
	Liver (rat)	Sodium malonate	100	5·4	32·0	16·6	11·5	2·7	—	62·8	72·4
	Tyrosinase (mushroom)	—	100	5·4	57·1	7·9	—	—	—	65·0	74·9
3	Liver (human)	—	63	193	14·4	13·3	14·8	2·3	<0·1	44·8	64·5
	Nil	—	63	193	1·7	67·7	—	—	<0·1	69·4	100
	Liver (human)	NaCN	63	193	6·1	22·3	20·9	0·9	<0·1	50·3	72·5
4	Placenta (human)	—	800	630	1·9	51·3	31·6	0·3	—	85·1	95·1
	Nil	—	800	630	1·4	88·1	—	—	—	89·5	100
	Placenta (human)	—	1600	1260	2·9	43·7	24·0	0·3	—	70·9	—
	Blood (human)	—	1600	1260	1·9	70·9	18·0	—	—	90·8	—

* Homogenate.

products formed *in vitro* from oestrone and to determine whether liver and mushroom tyrosinase gave rise to identical metabolites. Table 2 shows the behaviour of the ^{14}C compounds in the ether-extracted aqueous fractions under different physical conditions. Lowering the pH results in a considerable amount of the radioactivity passing into the ether layer on extracting either the liver or the tyrosinase aqueous fractions, which might indicate the presence of one or more acidic metabolites. These would exist in the un-ionized form at pH 1–2 and should therefore be more ether-soluble under these conditions. Butanol and ethanol remove about 50 % of the radioactivity at pH 7·4 from the aqueous fraction of the liver, but only a relatively small amount of ^{14}C passes into butanol from the corresponding tyrosinase fraction. The small quantity that is extracted can be accounted for by the partial solubility of water in this organic solvent.

Lowering the pH results in no further extraction of radioactivity by butanol from the tyrosinase solution, but with liver this solvent removes an amount (84·0 %) approximately equal to the sum of the amounts removed by ether from acid solution (33·9 %) and by butanol from neutral solution (53·1 %). This suggests the existence of another type of metabolite soluble at both acid and neutral pH which is formed by liver but not by the mushroom phenol oxidases.

A conjugate of oestrone or that of a closely related steroid metabolite must be ruled out unless it were able to resist hydrolysis by 7 % (w/v) HCl, since under these conditions no further ether-extractable radioactive material is formed. Beer & Gallagher (1955) have, however, presented evidence that glucuronosides may not be completely hydrolysed by refluxing with acid.

In the ethereal fractions of incubated liver and placenta the presence of unchanged oestrone and three more polar metabolites were revealed by chromatography followed by radioautography. Only the placental extracts were further examined since they contained considerably more ^{14}C than the liver ethereal fractions. Mixed chromatography with [16-^{14}C]oestradiol with the toluene–propylene glycol system showed the absence of this oestrogen and the presence of a metabolite more polar than oestradiol ($R_{oestradiol}$ 0·66), as well as another substance which had hardly moved from the starting line in 24 hr. Chromatography on the methylene chloride–formamide system (40 hr.) revealed that this latter compound was a mixture of two oestrone metabolites ($R_{oestriol}$ 0·91 and 0·33), with the faster-moving one differing from oestriol as confirmed by isotope-dilution analysis. All these ether-soluble compounds were also formed in the presence of cyanide. No further investigation was carried out on the ethereal fractions owing to the large number of oestrone metabolites that can theoretically exist and the difficulties involved in preparing any such oestrone analogue.

Finally, a result which confirms the findings *in vivo* of Valcourt *et al.* (1955) is the absence of detectable radioactivity in the carbon dioxide collected during the incubation of liver slices, showing that C-16 at least of the steroid nucleus remains intact. The evidence therefore is against rupture of ring D and the mushroom tyrosinase results are in favour of an initial attack in ring A of oestrone. The phenol oxidases are known to convert phenols into the corresponding *o*-quinones and there is no reason why a reaction of this type catalysed by similar oxidizing enzymes should not take place in liver tissue, with perhaps subsequent ring-opening and oxidation to a carboxylic acid. Some more circumstantial evidence in favour of

Table 2. *Examination of the ether-extracted aqueous fractions after incubation of* [16-^{14}C]*oestrone with liver and mushroom tyrosinase*

	Treatment	Percentage of ^{14}C removed from aqueous fraction
Liver		
	Acidified and extracted with ether	33·9
	Acidified and extracted with ether*	30·8
	Hydrolysed and extracted with ether	33·1
	Made alkaline and extracted with ether	9·2
	Evaporated to dryness and extracted with ethanol	50·0
	Extracted with butanol	53·1
	Acidified and extracted with butanol	84·0
Tyrosinase		
	Acidified and extracted with ether	51·4
	Extracted with butanol	11·6
	Acidified and extracted with butanol	53·4

* Aqueous fraction from the incubation of 100 μg. of oestrone. All other results for liver refer to the aqueous fraction of the incubation of 400 μg. of oestrone (see Table 1).

attack at ring A is provided by the recent isolation of 2-methoxyoestrone from human urine by Kraychy & Gallagher (1957). It suggests the conversion of oestrone into a catechol which might then be protected from rapid degradation in the body by methylation. Garst & Friedgood (1952) have obtained a similar type of compound from urine, for which they proposed an oestriol catechol or quinone structure. The formation of neutral metabolites from phenolic oestrogens *in vivo* (Valcourt *et al.* 1955; Beer & Gallagher, 1955) once more points to metabolic changes in ring A, whereas no trace of a marrianolic acid type of compound (Fieser & Fieser, 1949) formed by oxidative fission of ring D of oestrone has ever been detected in the living organism.

SUMMARY

1. Incubation of rat- and human-liver slices and mushroom tyrosinase with [16-^{14}C]oestrone results in the formation of water-soluble ether-insoluble metabolites, in high percentage yield, which do not appear on incubating oestrone with placental tissue or blood.

2. Cyanide, but not malonate, inhibits the formation of such oestrone metabolites by liver slices and also the adsorption or incorporation of ^{14}C by this tissue.

3. The nature of the oestrone-degradation products has been investigated and it has been shown that liver forms probably non-steroidal oestrogen metabolites which differ in part from those formed by mushroom tyrosinase.

4. No radioactive carbon dioxide was evolved during the incubation of [^{14}C]oestrone with liver.

5. Both liver and placenta are able to convert oestrone into ether-soluble metabolites even in the presence of cyanide, and with placenta these were shown to differ from either 17β-oestradiol or oestriol.

6. The evidence in favour of oestrone-degradation by initial attack in ring A is discussed.

This work was originally started at McGill University, Montreal, during the tenure of a Postdoctorate Fellowship and I should like to thank the National Research Council, Ottawa, for financial support and acknowledge with gratitude the advice and constant encouragement given to me by the late Professor R. D. H. Heard, F.R.S. (Canada). I am also grateful to him for a generous gift of radioactive oestrone and oestradiol, to Parke, Davis and Co. Ltd., Detroit, Mich., U.S.A., for oestriol and to the Royal Victoria Hospital, Montreal, for making available the human tissues. The project was continued at the Medical College of St Bartholomew's Hospital, where Professor A. Wormall, F.R.S., has provided excellent research facilities, and my thanks are also due to Dr E. D. Wills and other members of the Biochemistry Department for helpful suggestions. Miss A. Routledge gave valuable technical assistance.

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The Biochemistry of Rumen Protozoa

1. CARBOHYDRATE FERMENTATION BY *DASYTRICHA* AND *ISOTRICHA*

By B. H. HOWARD
Rowett Research Institute, Bucksburn, Aberdeenshire

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Of the various Protozoa found in the rumen of sheep and cattle, the holotrichous ciliates are represented by two genera: *Isotricha*, containing *I. intestinalis* and *I. prostoma*, and *Dasytricha*, containing only *D. ruminantium*. The two genera are closely related, the most obvious morphological difference being the greater size of the two *Isotricha* species. The biochemical properties of these organisms were first investigated by Oxford (1951) and Masson & Oxford (1951), who found that adding glucose to strained sheep-rumen liquor caused the holotrichs to sink to the bottom of the vessel as a dense white layer by virtue of the considerable amount of amylopectin synthesized and deposited inside the cells. Such suspensions, washed almost free of bacteria, were used by Sugden & Oxford (1952) in cultural studies, and by Heald, Oxford & Sugden (1952) and Heald & Oxford (1953) in manometric studies. The last-named authors found that mixed suspensions of the three species, in the presence of bactericidal concentrations of streptomycin, carried out a vigorous anaerobic fermentation of a number of sugars and polysaccharides. The chief products of the fermentation of glucose were acetic, butyric and lactic acid, carbon dioxide, hydrogen and amylopectin.

The first suggestion that the two genera might differ biochemically was made by Sudgen & Oxford (1952), who observed that when rice-starch grains were added to a mixed suspension of the holotrichs, only the isotrichs were seen to swallow the grains. The isotrichs were thus enabled to survive, whereas the *D. ruminantium* died. Gutierrez (1955) was the first to examine suspensions of either genus apart from the other. He separated organisms of the two genera from a mixture by taking advantage of differences in the sedimentation characteristics. By using these suspensions, it was shown that *D. ruminantium* was able to ferment cellobiose, but the isotrichs could not. Mould & Thomas (1957) observed that the amylases obtained from the two genera differed in electrophoretic mobility.

It is of obvious interest to investigate further these differences between the genera of rumen holotrichs. A method of obtaining pure suspensions of either genus is required which is more convenient than that of Gutierrez (1955), and which also eliminates the possibility of incomplete separation of the two types of organism.

The experiments in this Institute on removing fauna from sheep rumens (Eadie & Oxford, 1957) were designed partly toward this end. These workers were able to remove all holotrich Protozoa from the rumen and, since transfer of these organisms is solely from mouth to mouth (Becker & Hsiung, 1929), the treated sheep remained in that condition indefinitely if kept in isolation. It was further found possible to re-establish in such a sheep a culture of either *D. ruminantium* or mixed *Isotrichia* species by adding a suspension of the organisms, which had been individually separated under a dissecting microscope from a mixture of holotrichs obtained from another sheep. The population increased to a suitable level in 3–5 weeks. When the usual procedure for obtaining holotrich suspensions was applied to rumen liquor from such sheep, good yields of either *D. ruminantium* or *I. prostoma* plus *I. intestinalis* were obtained. Experiments with such suspensions are described here. This work has been already briefly reported (Howard, 1957*a*).

MATERIALS AND METHODS

Sheep containing a single genus of holotrich. The removal of fauna from, and establishment of *D. ruminantium* in, a single sheep has already been described (Eadie & Oxford, 1957). A mixture of *I. intestinalis* and *I. prostoma* was similarly established in the rumen of another fistulated sheep. Both sheep were initially fed on hay only, but as the animals began to lose weight and appetite, and the protozoal population fell, the diet was supplemented by a small proportion of grass cubes during the course of these experiments.

Preparation of protozoal suspensions. Samples of 1 l. of rumen liquor were collected in the morning about 2 hr. after feeding, and treated as described by Eadie & Oxford (1955). Unless stated otherwise, glucose was used to cause settling of the Protozoa, and the buffer solution of Sugden & Oxford (1952) was used for washing. By frequently changing the buffer, the isotrichs could be kept alive for over 48 hr., by which time the endogenous fermentation rate had fallen to a low level. The *D. ruminantium* suspensions, however, were invariably dead or dying after being kept overnight, and it was therefore necessary to use the

D. ruminantium suspensions on the day of collection, allowing only a few hours' starvation. The washed suspensions of Protozoa were sufficiently free from bacteria to make the use of antibiotics unnecessary (Heald & Oxford, 1953).

Substrates. Sugars were either Kerfoot's Biochemical Reagents or AnalaR; glucosides were supplied by L. Light and Co. Rice starch was a gift of Reckitt and Colman Ltd., Hull. Holotrich-starch grains were prepared as described in the next paper (Howard, 1959). Holotrich starch was defatted by boiling under reflux with two successive portions of 85% (v/v) methanol in water. Precipitated starch was made by dissolving the grains in hot water and precipitating with ethanol.

Manometric experiments. The same buffer solution was used in the Warburg cups as for washing the Protozoa; it includes 0·1% of $NaHCO_3$. The gas phase was N_2+CO_2 (95:5), freed from traces of O_2 by bubbling through Hungate's (1950) chromous sulphate reagent. The side arms contained 0·2 ml. of buffer solution in which was dissolved 10μmoles of sugar or glucoside or, for di- and trisaccharides, an amount yielding 10μmoles of fermentable sugar on hydrolysis. An exception was aesculin which, because of its relative insolubility, could be used at the rate of only 4μmoles/cup. Protozoal suspension (1 ml.) was placed in the main chamber of each cup immediately before gassing. Duplicate 1 ml. portions were analysed for total N. The total volume of fluid in each cup was 1·2 ml. Six manometers were used with each batch of Protozoa: two each to measure endogenous fermentation, glucose fermentation and test-substance fermentation. In the experiments designed to permit the analysis of galactose-fermentation products, the manometric procedure was modified as follows. The buffer solution used to suspend the organisms was the 'phosphate saline' of Heald & Oxford (1953), which contains no acetate or bicarbonate. The gas phase was O_2- and CO_2-free nitrogen. Warburg cups with two side arms were used; the first containing galactose (10μmoles in 0·2 ml. of buffer solution), and the second containing 10N-H_2SO_4 (0·2 ml.). The centre well contained 5N-NaOH (0·3 ml., carbonate-free). Acid was tipped in a control manometer at the beginning of the experiment, and in the others at the end, to stop the fermentation and to release CO_2.

Analytical methods. Total N in the protozoal suspensions was determined by a micro-Kjeldahl procedure. Reducing sugars were determined by Somogyi's (1945*a*) method, after clarifying with $ZnSO_4$ and baryta (Somogyi, 1945*b*). The aglucones produced during fermentation of the glucosides were detected by the colour reactions of Barnett, Ingram & Swain (1956). Paper chromatography of sugar solutions was as described by Howard (1957*b*). Lactic acid was determined by the method of Barker & Summerson (1941), and volatile acids by chromatography on buffered Celite columns (Bueding & Yale, 1951). Carbon dioxide in the alkali in the centre well of the Warburg cups was determined gravimetrically as $BaCO_3$. Storage polysaccharide in the cells was determined by reducing-sugar estimation after acid hydrolysis.

RESULTS

The results of a typical pair of manometric experiments, involving the fermentation of maltose by *D. ruminantium* and mixed isotrichs, are shown graphically in Fig. 1. The CO_2 constant, k_{CO_2}, was used to convert manometer readings into gas volumes, although the gas produced in the Warburg cups contained a small, and possibly variable, proportion of hydrogen. The reasons for using the CO_2 constant in this case have been discussed by Heald & Oxford (1953). In assessing the ease with which a substance was fermented, the rate of fermenta-

Table 1. *Rates of fermentation of carbohydrates by suspensions of* Dasytricha ruminantium *and* Isotricha *species*

See text for experimental conditions.

Substrate	*Dasytricha*		*Isotricha*	
	Rate of fermentation (%)*	Sugar used (%)†	Rate of fermentation (%)*	Sugar used (%)†
Galactose	70, 80, 67	96	0	1
Galactose‡	67	—	—	—
Maltose	33	52	0	0
Cellobiose	100	—	0	—
Aesculin	30, 50	§	4	§
Amygdalin	82, 93	—	13	—
Arbutin	60	§	28	§
Salicin	91	§	25	§
Sucrose	100	—	100	—
Raffinose	100	—	100	—
Xylose	0	1	0	—
Rice-starch grains	0	—	25	—
Holotrich starch (grains)	0	—	3·2	—
Holotrich starch (defatted grains)	0	—	3·3	—
Holotrich starch (precipitated)	0	—	1	—

* Compared to glucose as 100%. † As % of glucose used in same period.
‡ Protozoa collected from rumen liquor by adding galactose instead of glucose.
§ Aglucone detected in the solution at end of experiment.

Table 2. *Products of fermentation of glucose and galactose by* Dasytricha ruminantium

See text for experimental conditions.

Product	Moles of product/100 moles of sugar used		Atoms of C (% of C in total breakdown products)	
	Glucose*	Galactose†	Glucose*	Galactose†
Glucose in cells	82‡	28·1	—	—
Carbon dioxide	23	92·1	21·5	23·0
Acetic acid	7·0	28·0	13·1	14·0
Butyric acid	4·8	12·9	17·7	12·9
Lactic acid	17	66·5	47·6	50·0

* Calculated from Table 1 of Gutierrez (1955). † Present results. ‡ Calculated by difference.

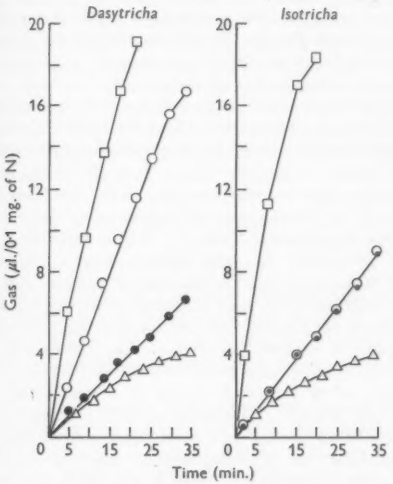

Fig. 1. Fermentation of sugars by the two genera of rumen holotrich Protozoa. For experimental conditions see text. ●, Endogenous fermentation; □, glucose; ○, maltose; △, glucosamine hydrochloride.

tion was compared with the endogenous fermentation and the fermentation of glucose, by portions of the same suspension. It was hoped in this way to allow for variations in metabolic activity between batches of Protozoa collected on different days. The rate of fermentation of a substance, corrected for the endogenous fermentation, could then be expressed as a percentage of the similarly corrected glucose-fermentation rate. A number of such values are shown in Table 1. Very high and very low values were reproducible between samples of Protozoa collected on different days, but where the rate of fermentation of a substance was intermediate the values sometimes differed markedly between samples.

In every case the rate of fermentation was linear during the period of observation, except for very rapidly fermented substances such as glucose, where exhaustion of substrate and developing acidity soon caused a falling-off in the rate of fermentation.

All the carbohydrates known hitherto as substrates for fermentation by rumen holotrichs are glucose, fructose and their derivatives. Galactose is the first exception to this generalization to be discovered, and it seemed of interest to investigate the products of metabolism of this sugar by *D. ruminantium*. An analysis of the contents of Warburg cups after such a fermentation is shown in Table 2. The reducing sugar present in acid hydrolysates of the cells before and after fermenting galactose was shown chromatographically to be almost wholly glucose; galactose was absent.

Mannose and glucosamine hydrochloride were not fermented by either type of holotrich, but were not metabolically inert, since a depression of endogenous fermentation was caused after a lag period. The effect of glucosamine hydrochloride is shown in Fig. 1; mannose produces a similar, but rather smaller, depression of endogenous fermentation. Microscopic examination at the end of the experiment showed the Protozoa exposed to mannose or glucosamine to be moribund, in contrast with the controls, which were normally active.

DISCUSSION

The conclusion, drawn from previous observations, that some biochemical differences exist between *Dasytricha* and *Isotricha* is confirmed by the present work. The fermentation of cellobiose by *Dasytricha*, but not by *Isotricha*, first observed by Gutierrez (1955) with organisms obtained from cattle in Pullman, Washington, U.S.A., has also been observed here with organisms from sheep. Whereas the *Dasytricha* studied in the present work fermented cellobiose approximately as rapidly as glucose, measurement of Gutierrez's (1955) fig. 1 suggests a fermentation rate for cellobiose of about 50% only, in the sense used in Table 1 above. Sugden & Oxford (1952) observed that adding

cellobiose to a mixed suspension of *D. ruminantium* and isotrichs prolonged the life of both genera. Since isotrichs alone cannot use cellobiose, it is evident that in this case they were profiting by the hydrolytic activity of *D. ruminantium*. The liberation into the medium of small amounts of cellobiase on the death and disintegration of *D. ruminantium* cells is the most likely explanation (see next paper, Howard, 1959). Eadie & Oxford (1955) described an abnormality of amylopectin storage that could be induced in rumen holotrichs while they were rapidly metabolizing a soluble sugar. When cellobiose was added to a mixed suspension, abnormality could be induced in *D. ruminantium* only, an observation explicable in the light of the present results.

Another example of the greater metabolic versatility of *Dasytricha* is the action of the two genera upon maltose. Here again, the isotrichs are unable to ferment this sugar, whereas *D. ruminantium* can do so, but not so quickly as glucose is fermented. When Heald *et al.* (1952) and Heald & Oxford (1953) tested mixed suspensions manometrically, no fermentation of maltose was observed. Apart from the low rate of maltose fermentation by mixed organisms which could be predicted from the data in Table 1, there is another factor which might have contributed to the diminution of activity. It has been noticed during the present experiments that when mixed suspensions are incubated for periods of about 48 hr., as in the experiments of Heald & Oxford (1953), the *D. ruminantium* tend to die off more rapidly than the isotrichs. A combination of these different factors might very well give rise to a suspension whose maltose-fermenting capacity is below what is measurable manometrically. The failure of Eadie & Oxford (1955) to induce abnormal amylopectin storage in either genus with maltose as substrate may be taken to indicate that fermentation approximately as rapid as that of glucose is required for the abnormality to be induced.

In fermenting the naturally occurring β-glucosides aesculin, amygdalin, arbutin and salicin, *Dasytricha* again has the advantage over *Isotricha*. In each case, the rate of fermentation by the former is several times that by the latter (Table 1). In contrast with its inability to ferment the β-glucoside cellobiose, *Isotricha* has an undoubted ability to make use of the glucose in the phenolic β-glucosides mentioned above, as is indicated not only by the gas production in the Warburg manometers but also by the detection of the aglucones in solution at the end of the experiment. The utilization of salicin for amylopectin synthesis by *D. ruminantium* but not *Isotricha* has already been noted by Gutierrez (1955); presumably the slow rate of fermentation by the latter does not allow of the accumulation of storage material.

When surveying infusoricidal compounds, Eadie, Mann & Oxford (1956) observed that not only was menthol (4 mM) very toxic to rumen holotrichs, but that both genera were killed by 6 mM-menthyl β-glucoside. The glucoside was toxic to the isotrichs even when no *D. ruminantium* were present, an observation which was inexplicable at the time, since it was concluded from the results of Gutierrez (1955) that no β-glucosidase was associated with *Isotricha*. The present work, showing that this genus does possess a definite small β-glucosidase activity, explains the apparent anomaly. In contrast with the toxicity of menthyl β-glucoside, no toxic effects of aesculetin, mandelonitrile, quinol or saligenin were noted in the present experiments, even though the concentration of aglucone that would be produced by complete hydrolysis of the glucosides is greater than 8 mM.

It was first observed by Sugden & Oxford (1952) and later by Gutierrez (1955) that *Isotricha* can ingest small starch grains, e.g. rice starch, but that the smaller *Dasytricha* cannot do so. That the rice-starch grains are truly fermented by the isotrichs but not by *D. ruminantium* is proved by the manometric experiments (Table 1). It is noteworthy that gas evolution in the manometers containing *Isotricha* commenced at the time of mixing; that is, the swallowing of, and biochemical attack on, the grains is very rapid. This agrees with the finding of Sugden & Oxford (1952) that the deposition of storage amylopectin granules was detectable within 5 min. of adding rice starch to a suspension of starved isotrichs. The same authors reported that granules of starch prepared from mixed rumen holotrichs were not swallowed or otherwise utilized by either *Isotricha* or *Dasytricha*. This was surprising, because the protozoal-starch grains are much smaller than rice-starch grains, and might be expected to be easily engulfed by *Isotricha*. This observation has been confirmed manometrically (Table 1). One possible explanation, that a lipid coating rendered the grains unpalatable, is excluded by the lack of activity of the Protozoa towards defatted grains. Eadie & Oxford (1955), contrary to the previous observation of Sugden & Oxford (1952), observed some ingestion of the protozoal-starch grains by *Isotricha*. It is clear that further investigation, both microscopical and biochemical, is required before these anomalies can be explained.

The products of galactose metabolism by *D. ruminantium* are identical with those formed from glucose by the same species (Table 2, columns 2 and 3). Particularly noteworthy is the conversion of a part of the galactose into glucose, as indicated by the increase in amylopectin content of the cells during fermentation. Perhaps because the galactose fermentation is slower than that of glucose, a much

smaller proportion is converted into amylopectin. Of the sugar converted into fermentation products proper, the proportions of the different products in the two cases are very similar (Table 2, columns 4 and 5). The most probable explanation of these results is that the galactose is first converted into glucose or a derivative. This hypothesis agrees with the results of studies of galactose metabolism by other organisms, where it has been shown (Leloir, 1955) that the following reactions are brought about by the action of the 'galactowaldenase' enzyme system: (1) uridine diphosphoglucose + galactose 1-phosphate ⇌ uridine diphosphogalactose + glucose 1-phosphate, (2) uridine diphosphogalactose ⇌ uridine diphosphoglucose.

The depression of fermentation caused by mannose and glucosamine, which was noted in the manometric experiments, agrees with the microscopic observations of Sugden & Oxford (1952), who found these two sugars, and also galactosamine, to be toxic to both genera of rumen holotrichs within 24 hr.

Further discussion of the present results will be found in the next paper (Howard, 1959).

SUMMARY

1. Suspensions of *Dasytricha ruminantium* and of mixed *Isotricha intestinalis* and *Isotricha prostoma* have been prepared from the rumen liquor of specially treated sheep, and used in manometric experiments.

2. The *D. ruminantium* suspensions, but not the isotrichs, were able to ferment galactose, maltose and cellobiose. Some naturally occurring β-glucosides were more readily fermented by *D. ruminantium* than by the isotrichs.

3. The products of galactose fermentation by *D. ruminantium* were the same as those formed from glucose by the same species.

4. Only the isotrichs could ferment rice-starch grains; neither genus could utilize starch grains from rumen holotrichs.

5. Previous observations on the rumen holotrichs, mostly made on mixtures of *Dasytricha* and *Isotricha*, are discussed in the light of these results.

I am grateful to my colleague Dr J. Margaret Eadie for making available to me the sheep used in these experiments, and for much advice on the handling of rumen Protozoa. My colleague Dr R. J. Pennington kindly performed the fatty acid analyses. Miss S. Bell, Miss C. M. Davidson and Mr W. Shand have assisted in this work.

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The Biochemistry of Rumen Protozoa

2. SOME CARBOHYDRASES IN CELL-FREE EXTRACTS OF *DASYTRICHA* AND *ISOTRICHA*

By B. H. HOWARD
The Rowett Research Institute, Bucksburn, Aberdeenshire

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The holotrich Protozoa of the rumen are known to possess a vigorous carbohydrate-fermenting system. In addition to glucose and fructose, a number of di-, tri- and poly-saccharides and glucosides can be fermented more or less readily (Heald & Oxford, 1953; Gutierrez, 1955; Howard, 1959). Cell-free extracts prepared by rupturing the protozoal cells have been found to contain invertase (Heald, Oxford & Sugden, 1952; Christie & Porteous, 1957) and amylase (Mould & Thomas, 1957). The last-named authors have also found in the extracts cellobiase, maltase and the enzymes responsible for the synthesis of starch (Mould & Thomas, 1958). They also achieved a partial separation of the two

genera concerned and showed that *Dasytricha* is much richer in cellobiase and maltase than is *Isotricha*. This paper reports a further investigation of the carbohydrases of these organisms, with the advantage of the facilities mentioned in the preceding paper (Howard, 1959) to examine the two genera *Dasytricha* and *Isotricha* separately. A part of this work has already been briefly reported (Howard, 1957*a*).

MATERIALS AND METHODS

Reagents and substrates. These were as mentioned in part 1 (Howard, 1959). In addition, the levan of *Pseudomonas mors-prunorum* ('bacterial levan') had been previously prepared in this Laboratory by Dr J. W. Porteous, and the laminaribiose was the gift of Dr W. A. P. Black, of the Institute of Seaweed Research, Inveresk, Midlothian. Samples of the three authentic fructosylsucroses were presented by Dr J. S. D. Bacon; the kestose was part of a gift to him from Mr P. H. Blanchard of the Tate and Lyle Research Laboratories.

Preparation of cell-free enzyme solutions. Suspensions of the Protozoa were prepared almost free from bacteria by the method of Eadie & Oxford (1955). The washed cells, freed from as much supernatant buffer solution as possible, were stored at −20°. To rupture the cells, the vessel containing the frozen mass was incubated at 38° for 2 hr. with occasional vigorous shaking. The milky suspension was centrifuged at 600 *g* for 30 min. and the opalescent supernatant was dialysed, first for a few hours against running tap-water, and finally overnight at 1° against distilled water. Centrifuging of the dialysed solution at 12 000 *g* for 30 min. yielded a clear solution of the enzymes, which was stored at 0° under toluene.

By repeated washing and centrifuging of the broken protozoal cells a clean suspension of holotrich-starch grains was obtained.

Assay of enzyme activity. The assay system contained 50 μmoles of substrate, 5 ml. of 0·2 M-sodium acetate–acetic acid buffer, pH 5, 1 ml. of an appropriate dilution of the enzyme and the whole was made up to 10 ml. with water. Hydrolysis of substrate was usually measured after incubation at 38° for 1 hr. Qualitative tests for hydrolysis were made by incubating enzyme and substrate together in melting-point tubes (Porter & Hoban, 1954), usually overnight, and spotting directly on to chromatogram papers which carried $HgCl_2$ on the starting line (Bealing & Bacon, 1953). Since the optimum pH values of the different enzymes of the two genera were unknown, the assays were conducted at an arbitrary value of pH 5, at which most carbohydrases are active. The differences in activity which were under examination were found to be so large that any error caused by not working at the optimum pH could be disregarded.

Analytical methods. Protein nitrogen in the enzyme preparations was determined by a modification of the method of Falconer & Taylor (1946). Aglucones formed from aesculin, arbutin and salicin were measured by the methods of Barnett, Ingram & Swain (1956). Sucrose hydrolysis was measured by the determination of reducing sugar (Somogyi, 1945). No deproteinization step was found to be necessary in the above determinations.

Use of glucose oxidase for detecting enzymes active against glucose-containing disaccharides. The method of glucose determination of Huggett & Nixon (1957) was used, crude glucose oxidase (Sigma Chemical Co., St Louis, Mo., U.S.A.) and peroxidase (C. F. Boehringer und Söhne, G.m.b.H., Mannheim-Waldhof, Germany) being employed. The tube containing the sample to be tested was immersed in boiling water for 10 min. to destroy enzymes before incubating with the glucose oxidase. The crude glucose oxidase used was free from lactase, melibiase and trehalase; it contained a trace of cellobiase, a little maltase and appreciable amounts of invertase.

Paper-chromatographic and electrophoretic methods. Descending chromatograms were run on Whatman no. 1 paper in butanol–acetic acid–water (4:1:5, by vol.). Benzidine–trichloroacetic acid (Bacon & Edelman, 1951) was used as a general spray, urea–phosphoric acid (Wise, Dimler, Davis & Rist, 1955) to reveal fructose derivatives and silver nitrate (Trevelyan, Procter & Harrison, 1950), with suitable controls, for the detection of reducing sugars. The conditions of paper electrophoresis were as described previously (Howard, 1957*b*), mobility with respect to sucrose (M_S) being calculated after correcting for electroendosmotic flow.

Large-scale treatment of sucrose by protozoal invertase. Sucrose (50 g.) dissolved in 0·2 M-sodium acetate–acetic acid buffer (pH 5, 200 ml.) was incubated at 38° with an enzyme solution from *Isotricha* (40 ml., containing 1·2 mg. of protein N). After 7 hr. the reducing power of the solution corresponded to 67% hydrolysis of the sucrose, and the reaction was stopped by treatment with $ZnSO_4$ and baryta. The mixture of sugars was separated by means of a charcoal column (Activated Charcoal, acid-washed, British Drug Houses Ltd.) (Crook & Stone, 1957). The concentration of ethanol in the eluent was raised stepwise to 10% (v/v), after which no further carbohydrate was eluted. Fractions from the column were collected over $BaCO_3$. After testing chromatographically, appropriate fractions were combined, concentrated *in vacuo*, filtered through a grade-5 sintered-glass filter and dried. The fructose content of these fractions was measured by the method of Roe, Epstein & Goldstein (1949), and total hexose by reducing-sugar determination after hydrolysis by 0·5% oxalic acid at 100° for 1 hr. The diazouracil test (Raybin test, for sucrose derivatives retaining an unsubstituted fructose group) was carried out as described by Feingold, Avigad & Hestrin (1956).

RESULTS

In order to make valid comparisons between different enzyme preparations, the quantitative results were calculated as μmoles of substrate hydrolysed by unit weight of protein nitrogen in the preparation. A number of such measurements are shown in Table 1. In the qualitative experiments few differences were observed between the two genera. Melezitose and xylobiose (4-*O*-β-xylosylxylose, Whistler & Tu, 1951) were not hydrolysed; raffinose was split into fructose and meliobiose, and laminaribiose was hydrolysed. Inulin and bacterial levan were hydrolysed; fructose was the only product of hydrolysis, and no oligosaccharides were detected. During hydrolysis

Table 1. *Hydrolytic activity of cell-free extracts of rumen holotrich Protozoa*

Substrate concentration, 5 mM, in 0·2 M-sodium acetate–acetic acid buffer, pH 5; temperature, 38°; results are expressed as μmoles of substrate hydrolysed in 1 hr./mg. of protein N.

Substrate	*Dasytricha ruminantium*	*Isotricha* spp.
Sucrose	910, 820, 1690	26 400, 4820
Maltose	30, 67	0·2
Cellobiose	130	1
Aesculin	890	14
Arbutin	470	12
Salicin	444	7
o-Nitrophenyl β-glucoside	65	1·4
Lactose	<0·1	<0·1
Melibiose	0·15	<0·1
Trehalose	0·15	<0·1

of amygdalin, glucose and a less-mobile substance, possibly gentiobiose, were seen on chromatograms. The *Dasytricha* extracts seemed to be more active than the *Isotricha* extracts in hydrolysing laminaribiose and amygdalin.

Oligosaccharide synthesis by the protozoal invertase

Enzyme solutions from *D. ruminantium* or mixed *Isotricha* spp. were incubated with sucrose (20 %, w/v) in acetate buffer, pH 5, under toluene. Samples taken at frequent intervals were examined chromatographically. The following observations were made with enzyme preparations from both genera. (1) Glucose and fructose were quickly produced and, as far as could be judged by inspection of the chromatograms, they were at all times in roughly equal amounts. (2) A number of other substances, each containing fructose, appeared during the course of the incubation; their concentrations in the mixture rose to a maximum and then declined. The last trace of these additional substances disappeared shortly before the last of the sucrose was hydrolysed, and the final products of enzyme action were glucose and fructose only. (3) The first oligosaccharide to appear, the last to disappear, and apparently the major synthetic product, was a non-reducing substance whose mobility on the paper chromatogram with respect to sucrose (R_s) was 0·43. The next spot to appear was also non-reducing, and had R_s 0·52. Two minor components were the last to appear; the more mobile (R_s 0·77) was a reducing sugar, the less mobile (R_s 0·21) was non-reducing. By comparison of the reaction of the test chromatogram towards the silver nitrate spray with the reaction of a spot of sucrose, it was considered that a trace of a reducing sugar having R_s 1·0 was also present. The order of disappearance of the synthetic products was the reverse of their order of appearance in the mixture. In a 20 % (w/v) sucrose solution containing 60 μg. of protein N of *D. ruminantium* enzyme/ml., hydrolysis was complete in 12 hr.

In a similar experiment where the enzyme was allowed to act upon a mixture of glucose and sucrose (10 %, w/v, each), the same spots corresponding to synthetic substances were seen on the chromatograms. In this case, however, the reducing sugar having R_s 0·77 was formed earlier and in larger quantity.

The action of the two enzyme preparations on solutions of raffinose (10 %, w/v), and raffinose + glucose (10 %, w/v, each) was determined. The only substances detectable with the urea–phosphoric acid spray were fructose and raffinose, together with a trace of a substance slightly less mobile than raffinose. No trace of sucrose could be detected.

Examination of fractions from charcoal column-chromatography of products of action of Isotricha *invertase on sucrose*

Seven main fractions were obtained; their properties were as follows.

Fraction 1. This was the aqueous eluate and contained only glucose and fructose.

Fraction 2. This was the disaccharide fraction and was eluted with 5 % (v/v) ethanol. In addition to sucrose, this fraction contained: (*a*) traces of a reducing sugar with mobility on the paper chromatogram approximately equal to that of sucrose (R_s 1·0); (*b*) a small amount of a reducing, fructose-containing sugar, R_s 0·77, indistinguishable from that formed by the action of the enzyme on sucrose and glucose together; (*c*) a non-reducing, fructose-containing sugar, found in a pure form in the next fraction. The disaccharide fraction was not examined further.

Fraction 3. This was eluted by 5 % (v/v) ethanol, and weighed 0·41 g. It was a non-reducing, Raybin-negative syrup and yielded only fructose and glucose on hydrolysis, in the ratio 2·17:1. It was homogeneous chromatographically and electrophoretically (R_s 0·43, M_s 0·72), and indistinguishable from kestose [*O*-α-D-glucopyranosyl (1 → 2)-*O*-β-D-fructofuranosyl-(6 → 2) β-D-fructofuranoside; 6^F-β-fructosylsucrose] when run on the same paper. When seeded with crystals of kestose, the syrup rapidly crystallized. Recrystallization from aqueous ethanol yielded fine white needles (0·24 g.), m.p. 143–145° (decomp.); $[\alpha]_D^{19} + 27{\cdot}3 \pm 0{\cdot}9°$ in water (*c*, 4·76). Albon, Bell, Blanchard, Gross & Rundell (1953) found kestose to have m.p. $145 \pm 2°$ (decomp.); $[\alpha]_D + 27{\cdot}3°$.

Fraction 4. This was eluted by 7·5 % (v/v) ethanol, and weighed 0·47 g. It was a non-reducing, Raybin-negative syrup, yielding on complete hydrolysis fructose and glucose only, in the ratio 2·1:1. It was homogeneous chromatographically

and electrophoretically (R_S 0·52, M_S 0·59), and indistinguishable from 1^F-β-fructosylsucrose [*O*-α-D-glucopyranosyl-(1→2)-*O*-β-D-fructofuranosyl-(1 → 2)-β-D-fructofuranoside] when run on the same paper. Seeding the syrup with crystals of 1^F-β-fructosylsucrose induced a slow crystallization. The crystalline product had m.p. 199–200° (decomp.); $[\alpha]_D^{19} + 26 \pm 3°$ in water (*c*, 1·1). Allen & Bacon (1956) found 1^F-β-fructosylsucrose to have m.p. 200° (decomp.); $[\alpha]_D + 28·6°$.

Fraction 5. This was a mixture (0·12 g.) of the substances found in fractions 4 and 6.

Fraction 6. This material (0·09 g.) was eluted by 7·5% (v/v) ethanol. It had the lowest chromatographic mobility of all the substances produced from sucrose by the protozoal invertase (R_S 0·21, M_S 0·6). It was Raybin-negative and on complete hydrolysis yielded only fructose and glucose in the ratio 2·85:1. It was not further examined.

Fraction 7. This material (0·45 g.) was eluted by 10% (v/v) ethanol. It was a syrup which did not crystallize, but was homogeneous chromatographically and electrophoretically (R_S 0·5, M_S 0·14), and was indistinguishable from authentic 6^G-β-fructosylsucrose [*O*-β-D-fructofuranosyl-(2 → 6)-*O*-α-D-glucopyranosyl-(1 → 2) β-D-fructofuranoside] when run on the same paper. On a dry-weight basis, the substance had $[\alpha]_D^{20} + 20·6 \pm 0·7°$ in water (*c*, 5·54). Allen & Bacon (1956) found $[\alpha]_D$ of 6^G-β-fructosylsucrose to be +21°. The substance gave a green colour in the Raybin test, and on complete hydrolysis yielded only fructose and glucose, in the ratio 2·16:1. When it was incubated in dilute acetic acid solution (Allen & Bacon, 1956) and the course of hydrolysis was followed chromatographically, the first products formed were fructose and sucrose. Later, glucose and a reducing, fructose-containing sugar were formed. This latter had R_S 0·8, indistinguishable chromatographically from the reducing sugar synthesized by the enzyme when incubated with sucrose and glucose.

DISCUSSION

It is clear from Table 1 that there are quantitative differences between the enzymes elaborated by the two genera of rumen holotrich Protozoa. Although the amounts of invertase in different samples of each genus differ considerably, *Isotricha* is clearly more active in producing this enzyme than *Dasytricha*, in the two populations which have been compared in this work. Mould & Thomas (1958) found the two genera to have roughly equal activities for equal weights of dried cells. In the present work, the protein nitrogen content of the cell-free extracts was made the basis of comparison, and this difference may explain the discrepancy between the two sets of results, particularly since the protein content of the protozoal cells can fall to a low proportion of the total weight during the deposition of amylopectin in the isolation procedure. It can also be seen from Table 1 that the β-glucosidase activity of mixed holotrichs is due mainly, and the cellobiase and maltase activities almost wholly, to *Dasytricha*. The differences between the genera in respect of these three enzymes are what would be expected from the observed differences in the abilities of the living organisms to ferment the same substrates. Thus it could be predicted from the results shown in Table 1 of the preceding paper (Howard, 1959) that maltase and cellobiase would be absent from *Isotricha*, but present, in moderate and large amounts respectively, in *D. ruminantium*. These predictions have been verified in the present experiments. *D. ruminantium* ferments a number of β-glucosides more rapidly than does *Isotricha* (Howard, 1959), and the cell-free extracts of the former organism show the expected greater hydrolytic activity towards the same substrates. On the other hand, the differences in invertase activity of the extracts are not reflected in any difference in fermenting activity, since *Dasytricha*, the genus poorer in this enzyme, is sufficiently rich in it to enable sucrose and raffinose to be fermented at the same rate as glucose. It will be noted that the activity of both genera towards *o*-nitrophenyl β-glucoside (Conchie, 1954) is much less than the respective activity towards the naturally occurring β-glucosides examined. The hydrolysis of laminaribiose and the production of glucose from amygdalin are probably other aspects of the β-glucosidase activity of the Protozoa.

The finding of only extremely small amounts of lactase, melibiase and trehalase in extracts of both genera agrees with the observations of Heald *et al.* (1952) that mixed living holotrichs were unable to ferment lactose, melibiose or trehalose. The apparent absence of either α- or β-galactosidase (melibiase and lactase respectively) makes the possession of a galactose-fermenting system by *D. ruminantium* somewhat puzzling. Free galactose is not present in the food of ruminants and, indeed, even in combined form it is by no means plentiful in the rumen. The only likely sources of galactose for a rumen-inhabiting micro-organism are the small amounts present in cereal polysaccharides (see, for example, Preece & Hobkirk, 1953) and the capsular polysaccharides of some types of rumen bacteria (Hobson & MacPherson, 1954). Recent observations have shown that under certain conditions *D. ruminantium* can feed upon rumen bacteria (Gutierrez & Hungate, 1957), but it seems unlikely that any galactose present in the polysaccharides of such bacteria can be made available to the protozoon by the action of its own enzymes. It

is possible that *Dasytricha* and *Isotricha* represent two successive stages in degeneration from a biochemically more versatile ancestral form which was able both to hydrolyse galactosides and to ferment the galactose so produced.

Cellobiose utilization by *D. ruminantium* is rather similar. It is known with certainty, from both microscopical and biochemical observations, that this organism does not attack cellulose. The possession of this enzyme can therefore be of use to the organism only if cellobiose diffuses into the rumen fluid from places where cellulose is undergoing bacterial decomposition. If this diffusion occurs at all, it must be very minute.

P. J. Heald and A. E. Oxford (personal communication, 1953) found that repeated fractional precipitation of extracts of rumen holotrichs with ammonium sulphate failed to alter the original ratio of invertase:levanase activity. Furthermore, D. L. Mould and G. J. Thomas (personal communication, 1957) found that electrophoresis of extracts of rumen holotrichs effected no separation of invertase and levanase activities. It seems probable therefore that the hydrolysis of inulin and bacterial levan, which proceeds by a stepwise removal of fructose units from the end of the polysaccharide chain, reflects a general β-fructosidase activity of the protozoal invertase. The enzyme resembles other fructosidases in being unable to hydrolyse the sucrose linkage in melezitose.

Like invertases from other sources, the protozoal invertase can act as a transfructosylase. The pattern of synthetic activity differs according to the source of the enzyme (Allen & Bacon, 1956), and it is possible from the results presented here to compare the protozoal invertase with those from yeasts, moulds and green plants. This is of particular interest because the synthetic activity of invertases of animal origin does not seem to have been examined previously. In this discussion, it will be taken as established that the three substances separated from the charcoal column as fractions 3, 4 and 7 are respectively kestose (6^F-β-fructosylsucrose), 1^F-β-fructosylsucrose and 6^G-β-fructosylsucrose. This conclusion is supported not only by the individual properties of the substances but also by the pattern of their elution from charcoal (cf. Bacon, 1954). The following identifications are highly probable, but not established with such certainty. (*a*) The reducing material which accompanies sucrose both on the paper chromatogram and on elution from charcoal is the complex of difructoses (component V of Bacon, 1954). (*b*) The reducing sugar with R_S 0·77 found in the disaccharide fraction from the charcoal column, and which is formed in greater quantity when free glucose is present in the enzyme–sucrose mixture, is fructosylglucose (component I of Bacon, 1954). (*c*) The spot of R_S 0·2 corresponds to component IV of Bacon (1954). It is probably a tetrasaccharide.

The properties of the protozoal invertase which are relevant to a comparison of transferring activity may be summarized as follows. (1) The main effect of the protozoal invertase is hydrolytic, i.e. under the conditions used in the present work, the yield of hydrolysis products (glucose and fructose) is much greater than the yield of synthetic products. (2) The chief products of transfructosylation are the three trisaccharides formed by attachment of a fructose residue at one of the three primary hydroxyl groups of sucrose. (3) Tetra- or higher oligo-saccharides are formed to only a very slight extent (fractions 5 and 6). (4) Transfer of fructosyl groups from raffinose to free glucose did not yield any chromatographically detectable sucrose, but rather a reducing disaccharide.

Allen & Bacon (1956) have compared the transferring activities of the invertases of yeasts, moulds and higher plants. The different properties of these invertases are summarized in Table 2, together with those of the protozoal invertase. It is clear that, in each of the four respects mentioned above, the protozoal invertase resembles that of yeast rather than those of moulds or green plants. Although similar qualitatively to the yeast invertase the protozoal enzyme seems to differ quantitatively; the overall yield of synthetic products was rather less than can be obtained by the use of the yeast invertase. The kinetic studies of Christie & Porteous (1957) also suggest that there are differences between the yeast and protozoal invertases.

Table 2. *Comparison of invertases from different sources*

	Source of enzyme			
	Rumen Protozoa	Yeast	Moulds	Green plants
Main action of enzyme	Hydrolysis	Hydrolysis	Transfer	Hydrolysis
Number of fructosylsucroses formed	3	3	2	2
Higher oligosaccharides	Little	Little	Much	None
Fructosylglucoses formed	No sucrose, component I*	No sucrose, component I*	Sucrose	Trace of sucrose

* Bacon (1954).

SUMMARY

1. Cell-free extracts, nearly free from bacteria, have been prepared from suspensions of the two genera of rumen holotrich Protozoa, *Dasytricha* and *Isotricha*.

2. The ability of these extracts to split a number of di-, tri- and poly-saccharides has been investigated. Hydrolytic activity of the extracts corresponded to the ability of the living Protozoa to ferment the substrates.

3. *Dasytricha ruminantium* extracts contained appreciable cellobiase and β-glucosidase activity, and moderate maltase activity. Extracts of mixed *Isotricha intestinalis* and *I. prostoma* contained hardly any maltase, a trace of cellobiase and a small amount of β-glucosidase. Neither genus gave extracts able to hydrolyse lactose, melibiose, trehalose, melezitose or xylobiose.

4. Considerable invertase activity was found in the protozoal extracts, especially those from *Isotricha*. Sucrose, raffinose, inulin and a bacterial levan were all hydrolysed, but not melezitose. The transferring activity of the invertase was examined, and some products of transfructosylation were isolated and identified. The protozoal invertase resembles most closely that of yeast.

It is a pleasure to acknowledge my indebtedness to Dr J. S. D. Bacon, Macaulay Institute for Soil Research, Aberdeen, without whose advice and gift of samples the work on the protozoal invertase could not have been completed. I am also grateful to my colleague Dr G. N. Festenstein for advice on the use of glucose oxidase, and to Miss C. M. Davidson and Mr W. Shand for their experimental assistance.

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The Formation of Mercapturic Acids

1. FORMATION OF MERCAPTURIC ACID AND THE LEVELS OF GLUTATHIONE IN TISSUES

BY M. M. BARNES, SYBIL P. JAMES AND P. B. WOOD
Physiology Department, The Medical School, University of Birmingham

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Since Baumann & Preusse (1879) and Jaffe (1879) showed that the administration of monohalogenobenzenes to the dog led to the excretion of mercapturic acids [*N*-acetyl-*S*-(*p*-halogenophenyl)-L-cysteines] many other mercapturic acids have been isolated from the urines of several species. These compounds may be classified in four types as shown in Table 1 and are, in effect, formed by elimination between precursor and acetylcysteine of 2H (type I), halogen hydracid (type II), HNO_2 (type III) or $HO_3S{\cdot}CH_3$ (type IV). The suffix *a*, *b* or *c* to the type numbers indicates an aryl, alkyl or aralkyl compound.

Baumann & Preusse (1879) and Jaffe (1879) suggested that mercapturic acids were produced in urine from an acid-labile compound. Boyland, Sims & Solomon (1957) and Boyland & Sims (1958) have shown that naphthylmercapturic acid is an

artifact formed by decomposition of a compound, probably *N*-acetyl-*S*-(1:2-dihydro-2-hydroxynaphthyl)-L-cysteine, which is excreted in the urine of several animals dosed with naphthalene. A reduction of the aromatic ring during the formation of mercapturic acid had been postulated by Rhode (1923), who suggested that *S*-(4-bromo-2-hydro-1-hydroxyphenyl)cysteine was an intermediate in the conversion of bromobenzene into bromophenylmercapturic acid. Knight & Young (1958) have obtained similar evidence for premercapturic acids from precursors of type I but not for benzyl chloride. In this Laboratory we have not been able to detect a premercapturic acid from 3:4-dichloronitrobenzene (unpublished result). It would seem, therefore, that mercapturic acids may be formed from precursors of type I by a different process from that by which mercapturic acids are formed from precursors of type II. It may be relevant that type I precursors yield mercapturic acids by, in effect, deprotonation whereas in types II, III and IV the elimination of a molecule of an acid is involved.

Although the details of the formation of mercapturic acid have not been elucidated it is usually held that the precursor is converted into an *S*-cysteine derivative which is then acetylated. Indirect evidence of this was obtained by several workers (e.g. Stekol, 1938; Zbarsky & Young, 1943; West, Mathura & Black, 1951; West & Mathura, 1954) who administered *S*-substituted cysteines to animals and isolated the corresponding mercapturic acids from the urine. More direct evidence was obtained by Mills & Wood (1956), who detected *p*-iodophenylcysteine as an intermediate in the formation of the mercapturic acid from *p*-iodobenzene by rat-liver slices and in the liver after oral administration of iodobenzene to rat.

The immediate source of the cysteine required for conjugation with the mercapturic acid precursor has not been established. Possible sources are dietary cysteine, cysteine residues of tissue protein, and glutathione. Stekol (1937*a–d*, 1939*a*) concluded that the addition of sulphur-containing amino acids or glutathione to the food of animals maintained on a balanced diet had no effect on the formation of mercapturic acid but that the addition of these acids augmented the synthesis in rats and dogs maintained on a low-sulphur diet. Simultaneous measurements of the nitrogen and mercapturic acid excretion led Stekol to suggest that the augmentation observed was a result of replenishment of tissue sulphur rather than evidence of the direct union of the mercapturic acid precursor with dietary amino acids. Stekol's conclusion was based on the results of estimations carried out on 24 hr. urine samples. Barnes & James (1957) used detailed excretion curves, based on the analysis of frequent successive urine samples, and showed that the administration of cysteine, cystine or methionine did not increase the rate of synthesis of mercapturic acid in the rabbit. Gutmann & Wood (1950) showed that less than 10% of [^{35}S]cysteine administered simultaneously with bromobenzene to rats was excreted as *N*-acetyl-*S*-(*p*-bromophenyl)-L-cysteine. Marsden & Young (1958) also found that only a small proportion of ^{35}S given as L-[^{35}S]cystine was excreted as 1-naphthyl[^{35}S]mercapturic acid by rats dosed with naphthalene. There is therefore evidence that dietary sulphur-containing amino acids are not the immediate source of more than a small fraction of the cysteine used in mercapturic acid formation.

Stekol (1937*a*; 1939*b*) and Smith, Spencer & Williams (1950) favoured the view that the first stage in the formation of a mercapturic acid consisted in the conjugation of the precursor with

Table 1. *Types of mercapturic acid precursors*

Type	Precursor	Examples	Group replaced by acetylcysteyl	Reference
I	Aromatic hydrocarbon or halogenobenzene	Benzene	H	Zbarsky & Young (1943)
		Chlorobenzene	H	Jaffe (1879)
		Naphthalene	H	Bourne & Young (1934)
II*a*	Halogenonitrobenzene	2:4-Dichloronitrobenzene	Cl	Bray, James & Thorpe (1957)
		p-Fluoronitrobenzene	F	Bray, James & Thorpe (1958*a*)
II*b*	Halogenoparaffin	Bromoethane	Br	Thomson, Maw & Young (1958)
		Bromobutane	Br	Bray & James (1958)
II*c*	ω-Halogenoalkylbenzene	Benzyl chloride	Cl	Stekol (1938)
		Phenethyl bromide	Br	Bray *et al.* (1958*b*)
III*a*	Halogenonitrobenzene	Pentachloronitrobenzene	NO_2	Betts, James & Thorpe (1955)
		2:3:5:6-Tetrachloronitrobenzene	NO_2	Bray, Hybs, James & Thorpe (1953)
III*b*	Nitroparaffin	Nitrobutane	NO_2	This paper
IV	Ester	Ethyl methanesulphonate	$HO_3S{\cdot}CH_3$	Roberts & Warwick (1957)

tissue protein. Stekol (1938, 1939*b*) cited as evidence the observation of Landsteiner & Jacobs (1936) that benzyl chloride produced sensitization in the guinea pig. The fact that this animal excretes very little mercapturic acid (see Bray, Franklin & James, 1959), however, detracts from the value of this argument, unless the reason for the small excretion is that any mercapturic acid formed is deacetylated or otherwise changed before excretion. Mills & Wood (1956) isolated a protein, containing radioactive material, from the liver of a rat which had been given [^{131}I]iodobenzene, but the possibility that the radioactivity was due to adsorbed material not removed by washing, rather than to the formation of a conjugate, was not excluded. These workers also prepared, by the action of diazotized *p*-iodoaniline on serum protein, a product which yielded *p*-iodophenylcysteine on hydrolysis. When the product was administered orally or intravenously to rats it was converted into *p*-iodophenylmercapturic acid, so it is possible that tissue protein may provide some of the cysteine required for mercapturic acid formation. However, the large amounts of mercapturic acid excreted after the administration of some mercapturic acid precursors to the rabbit, and the rapid rate at which they are formed, make it improbable that the tissue protein could furnish all the cysteine required for conjugation. Thus if liver protein is synthesized at the rate of 1·9 g./kg. of rabbit/day (Henriques, Henriques & Neuberger, 1955) about 2 mg. of protein S/2·5 kg. rabbit/hr. will be formed. Mercapturic acid can be formed from benzyl chloride at the rate of about 40 mg./2·5 kg. rabbit/hr. (Barnes & James, unpublished work) and this would require about 5 mg. of S/2·5 kg. rabbit/hr. Furthermore, Anson (1940, 1941), Anson & Stanley (1941) and Barron (1951) reported that not all the –SH groups of tissue protein are reactive and a proportion may not be available for combining with the foreign molecule.

The possible role of glutathione in furnishing the three amino acids utilized for conjugation with foreign molecules was considered by Waelsch (1930) and, although later evidence (Waelsch & Rittenberg, 1941) excluded the possibility that the sole source of glycine for hippuric acid formation was glutathione, the possibility that the cysteine residue was used in the formation of mercapturic acids has not been similarly excluded (Waelsch, 1952). Stekol (1940, 1941) gave benzyl- and *p*-bromobenzyl-glutathione to rats and isolated benzyl- and *p*-bromobenzyl-mercapturic acids from the urine. This is not unequivocal evidence that the mercapturic acid precursor combines with glutathione in the body since the substituted glutathione may have been hydrolysed in the gut (see Bray *et al.* 1959) and there is no proof that the glutathione conjugate itself was absorbed. Nakashima (1934) showed that the glutathione content of the eye lens and of the liver was depleted 24 hr. after the administration of naphthalene to the rabbit. Yamamoto (1940) related the fall in the level of liver glutathione in the rabbit dosed with bromobenzene to the excretion of *p*-bromophenylmercapturic acid. Binet & Wellers (1951) observed a decrease in the level of glutathione in the livers and kidneys of rats given bromobenzene. Snyder & Cornatzer (1958) showed that the amounts of acid-soluble –SH compounds, but not the protein sulphur-containing amino acids, of rat liver decreased after administration of bromobenzene.

Evidence for the participation of glutathione in the formation of mercapturic acids has been given in preliminary communications by Barnes & James (1957) and Bray & Franklin (1957) and some details of this work are now reported. This paper describes the effect of the administration of typical mercapturic acid precursors upon the level of glutathione in tissues of the rat and rabbit.

MATERIALS

All melting points are uncorrected.

Chlorobenzene, benzyl chloride, *p*-chlorobenzyl chloride, 1-bromobutane, 3:4-dichloronitrobenzene and butyl chloral hydrate were purchased. 2:4:6- and 3:4:5-Trichloronitrobenzenes were prepared by the method of Holleman & Haeften (1921) and 2:3:4:6-tetrachloronitrobenzene as described by Betts, James & Thorpe (1955). 1-Nitrobutane, b.p. 151°, was prepared by the interaction of 1-bromobutane and silver nitrite. It contained 0·8% of Br (i.e. 98% pure). Glutathione and oxidized glutathione were purchased from Boehringer und Söhne G.m.b.H., Mannheim.

METHODS

Animals and diet. Female rats (approx. 200 g.) were used for the determination of tissue glutathione. For excretion experiments they were housed in metabolism cages set over a funnel which was arranged over a fraction-cutter (Bray, Thorpe & White, 1951). The animals were maintained on a diet of rat cubes and water. The rabbits used were does (about 2·5 kg.) maintained on rabbit pellets and water. All doses were administered by stomach tube as suspensions in water.

Determination of mercapturic acid. For mercapturic acids formed from chlorobenzene, 2:4:6-trichloronitrobenzene, 2:3:4:6-tetrachloronitrobenzene and butyl chloral hydrate the modified Stekol (1936) method (Betts *et al.* 1955) was used; for those from benzyl chloride, *p*-chlorobenzyl chloride, 1-bromobutane and 1-nitrobutane the nitroprusside method (Bray, James & Thorpe, 1958*b*) was employed. For the last two the final concentration of NaOH used for the hydrolysis was increased to 4N and the time was 1·5 hr. Mercapturic acids formed from 3:4-dichloronitrobenzene and 3:4:5-trichloronitrobenzene were determined by the colorimetric method of Bray, James & Thorpe (1956).

The maximum rates of excretion of the mercapturic acid were measured by determining the concentration of mercapturic acid in the urine produced during the period from 2 to 6 hr. after dosing. During this period the excretion curves are nearly linear (see Fig. 1) and the rate of excretion is approximately constant. Urine was expelled from the bladder by gentle pressure 2hr. after the dose had been given. This urine was discarded. The rats were then given water (3 ml.) by stomach tube and from this time the urine was collected. A further 3 ml. of water was given after 5 hr. After 6 hr. the urine was expelled from the bladder as before and the combined samples were analysed. Usually six rats were used for each compound studied and a correction was made for base-line excretion of material reacting with the reagents by deducting one-sixth of the average normal 24 hr. values for undosed rats from the amount excreted by experimental rates during the 4 hr. In some experiments complete excretion curves of the mercapturic acid were obtained by analysis of successive urine samples for longer periods.

Determination of glutathione. The glutathione (GSH) and oxidized glutathione (GSSG) content of blood was determined by the iodometric method of Woodward & Fry (1932). In early experiments the GSH content of the tissues was determined by the enzymic method of Woodward (1935) as modified by Bhattacharya, Robson & Stewart (1955). In later experiments the method of Martin & McIlwain (1959) was used for GSH and GSSG. In this method GSSG is reduced by preincubation with the glutathione reductase of yeast.

Plan of experiments and collection of tissues

Rats. The dose, or in control experiments water alone, was administered to rats which had been starved for 19 hr. The animals were killed after the required time by a blow on the back of the neck. Blood was collected from the nostrils into potassium oxalate (37·5 mM), deproteinized and analysed for glutathione. The liver, kidneys and small intestine were quickly removed. A sample of the liver (about 1–2 g.) was separated, chopped up and placed in ice-cold 3% (w/v) sulphosalicylic acid (10 ml.) in a tared centrifuge tube. Whole kidneys were treated in the same way. The small intestine was slit longitudinally and the contents were washed out with water and discarded. The intestine was finely chopped and immersed in sulphosalicylic acid as with the liver and kidney. The tissues were homogenized with a Teflon pestle, diluted with 3% sulphosalicylic acid (10 ml.) and centrifuged. The supernatant was separated off. The residue was mixed with a further 10 ml. of 3% sulphosalicylic acid and centrifuged. Samples of the combined supernatants were used for determination of GSH and GSSG.

For the continuous injection experiments (see Fig. 2) the rat was kept in a cylinder of wire mesh, which prevented undesirable movement of the animal. The cylinder was held in a polythene funnel cut to fit it. The appropriate isotonic fluid was injected continuously into the caudal vein of the rat through fine polythene tubing which had been inserted while the tail was anaesthetized with procaine (cf. Kellogg, Burack & Isselbacher, 1954). The fluid was kept in a 50 ml. glass syringe held rigidly in a horizontal position. The plunger was fitted with a cap and wires so arranged that the plunger could be gradually drawn in by winding the wire on a slowly rotating spindle operated by an electric motor with a 10^6:1 reduction gear. The spindle was 'stepped' in several diameters so that the rate of movement of the plunger could be varied. When the wire was wound on a step of 1 cm. diameter the plunger was drawn in at the rate of 6 mm./hr. This apparatus is a modification of a device shown to us by Dr R. Keeler. Under the continuous injection of fluid the rat passed urine at frequent and fairly uniform intervals (see Fig. 2). After each sample the rat and funnel were washed with a small amount of water which was combined with the urine sample.

Rabbits. Blood samples (about 3 g.) were collected from an ear vein directly into 25 ml. of 37·5 mM-potassium oxalate.

RESULTS

Isolation of mercapturic acids from rat urine

N-Acetyl-S-(2-chloro-4-nitrophenyl)-L-cysteine. The urine of three rats which had each received 150 mg. of 3:4-dichloronitrobenzene was acidified and extracted with ether. *N*-Acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine crystallized from the extract. After recrystallization from aqueous ethanol it had m.p. 192°, alone and in admixture with an authentic specimen. Yield 100 mg. The spectrum was in agreement with that recorded for the synthetic material (Bray, James & Thorpe, 1957).

N-Acetyl-S-(2:3:4:6-tetrachlorophenyl)-L-cysteine. Evaporation of the solution obtained by continuous ether extraction of the urine of six rats which had each received 75 mg. of 2:3:4:6-tetrachloronitrobenzene gave crystals of *N*-acetyl-*S*-(2:3:4:6-tetrachlorophenyl)-L-cysteine. After recrystallization from aqueous ethanol the crystals (90 mg.) had m.p. 194°, alone and in admixture with a specimen isolated from rabbit urine (Betts *et al.* 1955); $[\alpha]_D^{24}$ was $+21 \pm 2°$ in ethanol (*c*, 0·66).

Extent of formation of mercapturic acid in the rat

The extent of the formation of mercapturic acid after administration of examples of precursors of types I, II and III to the rat is given in Table 2, from which it can be seen that the rat closely resembles the rabbit in its ability to form mercapturic acid from typical precursors. The maximum rates of excretion of some of the mercapturic acids during the period from 2 to 6 hr. after administration of the dose, the nearly linear part of the excretion curve, are given in Table 3. Fig. 1 shows typical excretion curves over longer periods obtained by the analysis of consecutive urine samples from rats given benzyl chloride, 3:4-dichloronitrobenzene and 3:4:5-trichloronitrobenzene.

Glutathione levels of tissues

Liver. The amounts of GSH and GSSG found in the liver of rats are given in Table 4. The administration of water by stomach tube to a rat had a

Table 2. *Excretion of mercapturic acid by the rat*

Values are means with ranges in parentheses; the superior figures indicate the number of experiments. Doses for the rabbit were of the same order as for the rat. Samples of urine were collected until excretion of mercapturic acid could no longer be detected (usually 24 hr.).

Precursor	Type	Dose for rat (μmoles/100 g.)	Mercapturic acid excreted (% of dose) Rat	Rabbit
Chlorobenzene	I	187	18 (15–23)[3]	20*
Benzyl chloride	II*c*	158	27 (20–32)[4]	49†
p-Chlorobenzyl chloride	II*c*	124	37 (21–47)[3]	23†
1-Bromobutane	II*b*	175	44 (21–64)[5]	45‡
1-Nitrobutane	III*b*	165	63 (62–64)[3]	23‡
3:4-Dichloronitrobenzene	II*a*	182	19 (4–29)[12]	45§
3:4:5-Trichloronitrobenzene	II*a*	110	24 (21–29)[3]	24§
2:4:6-Trichloronitrobenzene	III*a*	110	2 (0–4)[3]	<1§
2:3:4:6-Tetrachloronitrobenzene	III*a*	144	32 (29–38)[3]	37‖
2:3:5:6-Tetrachloronitrobenzene	III*a*	144	11 (8–14)[5]	14¶
Benzyl alcohol	—	171	0[3]	2†
Butyl chloral hydrate	—	65	0[3]	—

* Spencer & Williams (1950).
† Bray *et al.* (1958*b*). The value for benzyl alcohol is probably not significant.
‡ Unpublished results. § Bray *et al.* (1957). ‖ Betts *et al.* (1955).
¶ Only about 34% of this compound is absorbed by the rabbit and 76% by the rat.

Table 3. *Maximum rates of excretion of mercapturic acid by the rat*

Successive samples of urine were collected during the period from 2 to 6 hr. after dosage as described under Methods. Rates for this period are given as means with ranges in parentheses; superior figures indicate the number of experiments.

Precursor	Dose (μmoles/100 g.)	Rate (μmoles/100 g./hr.)
Chlorobenzene	169	3·5 (1·2–5·9)[6]
Benzyl chloride	158	5·8 (3·5–7·8)[6]
p-Chlorobenzyl chloride	124	2·9 (1·8–3·8)[6]
1-Bromobutane	157	4·9 (2·3–7·4)[6]
3:4-Dichloronitrobenzene	130	1·0 (0·4–1·9)[6]
3:4-Dichloronitrobenzene	182	2·1 (1·1–2·9)[6]
3:4:5-Trichloronitrobenzene	110	2·0 (0·9–2·5)[6]
2:3:4:6-Tetrachloronitrobenzene	144	3·2 (2·6–4·3)[5]

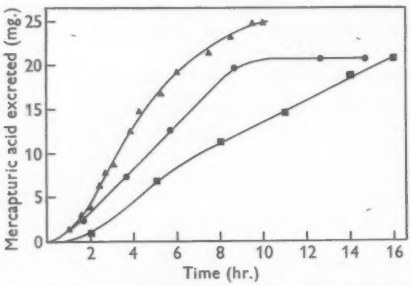

Fig. 1. Excretion of mercapturic acid by the rat. The dose was given at 0 hr. and water (3 ml.) was given 2-hourly. Doses were: ▲, benzyl chloride, 158 μmoles/100 g.; ●, 3:4:5-trichloronitrobenzene, 110 μmoles/100 g.; ■, 3:4-dichloronitrobenzene, 130 μmoles/100 g.

pronounced effect on the relative proportions of GSH and GSSG. If the rats were killed immediately after water had been given, the GSH level was about half the amount found when rats which had not been given water were killed; there was a corresponding rise in the GSSG level and the total glutathione was approximately constant.

When mercapturic acid precursors were administered to rats there was usually a significant fall in the glutathione levels which reached a minimum value with most compounds about 4 hr. after the administration of the dose. This is most clearly seen where the dose of precursor exceeded 130 μmoles/100 g. The results with doses below 130 μmoles/100 g. are less striking, although significant for at least one time period. The effect of dose level is seen with 3:4-dichloronitrobenzene, with which for a dose of 130 μmoles/100 g. there is a marked fall in GSH level only at 2 hr. after dosage; at 180 μmoles/100 g. the fall in GSH level is pronounced even 6 hr. after dosage. The fall in glutathione levels is particularly great with benzyl chloride and chlorobenzene. In most experiments the glutathione content after 6 hr. suggests the beginning of a return towards the normal value. After about 10 hr. the excretion of mercapturic acid is usually approaching completion (see Fig. 1),

Table 4. *Effect of mercapturic acid precursors on levels of glutathione in rat liver*

Doses suspended in 3 ml. of water were administered at 0 hr. to rats which had fasted for 19 hr. Results are expressed as means with ranges in parentheses; the superior figure indicates the number of experiments. The total glutathione values from dosed animals were all significantly lower by *t* test than the corresponding control values, except those for benzyl alcohol (*P* 0·2 and 0·3 for 2 and 6 hr. respectively) and butyl chloral hydrate (*P* 0·2 for 0·5 hr.). The water control values for 0·5, 2, 4 and 6 hr. against 0 hr. gave *P* 0·2, 0·2, 0·5 and 0·1 respectively. The 6 hr. values for bromobutane and 3:4-dichloronitrobenzene gave *P* 0·05 and 0·02.

Compound administered	Dose (μmoles/100 g. of rat)		Glutathione (mg./100 g. of liver) after 0 hr.	0·5 hr.	2 hr.	4 hr.	6 hr.
None	—	GSH	122 (114–134)[4]	—	—	—	—
		GSSG	48 (39–61)[4]	—	—	—	—
Water only	—	GSH	79 (59–112)[4]	120 (113–139)[6]	163 (139–181)[5]	140 (117–149)[6]	133 (113–169)[12]
		GSSG	110 (91–143)[4]	53 (47–57)[6]	14 (0–39)[6]	35 (23–49)[5]	34 (25–39)[4]
Chlorobenzene	169	GSH	—	—	52 (37–75)[6]	23 (13–28)[4]	21 (16–32)[4]
		GSSG	—	—	14 (0–23)[5]	8 (6–13)[4]	5 (2–6)[4]
Benzyl chloride*	158	GSH	—	38 (11–63)[11]	16 (0–35)[11]	20 (0–49)[11]	27 (10–57)[5]
		GSSG	—	21 (4–35)[5]	4 (0–10)[6]	15 (0–28)[5]	—
p-Chlorobenzyl chloride	124	GSH	—	—	105 (63–196)[7]	70 (30–136)[8]	100 (60–151)[5]
1-Bromobutane	157	GSH	—	—	67 (52–86)[4]	52 (32–74)[4]	84 (55–127)[6]
		GSSG	—	—	18 (7–32)[4]	20 (8–29)[4]	27 (13–38)[6]
1-Nitrobutane	165	GSH	—	—	—	63 (36–81)[4]	—
		GSSG	—	—	—	38 (26–54)[4]	—
3:4-Dichloronitrobenzene	130	GSH	—	183 (165–195)[8]	95 (60–151)[8]	127 (60–148)[8]	137 (102–152)[5]
	182	GSH	—	—	80 (36–114)[6]	67 (34–99)[6]	89 (66–110)[4]
		GSSG	—	—	31 (17–50)[6]	22 (12–42)[5]	44 (35–53)[4]
3:4:5-Trichloronitrobenzene†	110	GSH	—	157 (114–175)[6]	118 (70–162)[6]	95 (70–115)[6]	85 (44–121)[6]
2:4:6-Trichloronitrobenzene	110	GSH	—	—	161 (130–199)[5]	140 (127–154)[8]	—
2:3:4:6-Tetrachloronitrobenzene	144	GSH	—	—	75 (58–89)[4]	40 (20–58)[4]	46 (20–56)[4]
		GSSG	—	—	22 (10–34)[4]	16 (10–18)[4]	17 (10–20)[4]
Benzyl alcohol	171	GSH	—	—	114 (108–121)[4]	102 (87–111)[6]	96 (80–110)[6]
		GSSG	—	—	44 (37–49)[4]	33 (23–42)[4]	53 (31–65)[6]
Butyl chloral hydrate	65	GSH	—	124 (102–139)[6]	200‡ (148–274)[6]	—	—
		GSSG	—	35 (29–46)[6]	—	—	—

* The GSH values after 10 hr. were 70 (40–98)[6].

† The values after 18 hr. were 186 (180–190)[3].

‡ After 1 hr. This value is significantly higher than the control 0·5 hr. value for GSH (*P* 0·01) but not significantly different from the 2 hr. control for GSH (*P* 0·1) or the 0·5 hr. control for GSH + GSSG (*P* 0·2).

so that the period of decrease in the level of glutathione in liver is approximately coincident with the period of mercapturic acid excretion.

The amounts of glutathione found in the livers of rats given 2:4:6-trichloronitrobenzene were not significantly lower than those from undosed control animals. This compound forms only very small amounts of mercapturic acid (Table 2). Two compounds which are not mercapturic acid precursors are included in Table 4. So far as is known the metabolism of benzyl alcohol does not involve any reaction with a cysteine derivative. Of the results with this compound for total glutathione in Table 4 only those for 4 hr. are significantly lower (P 0·01) than those for the controls. Butyl chloral hydrate was chosen because, although it does not form mercapturic acid (Table 2), it is a compound which readily loses chloride and probably combines with –SH compounds in rabbit-liver extracts to an extent comparable with that for benzyl chloride (Bray, Thorpe & Vallance, 1952). It is doubtful whether this compound decreases the level of glutathione, but because of its anaesthetic properties the dose given was much smaller than that for the other compounds.

Kidney and small intestine. The glutathione content of these tissues after rats had been dosed with 3:4:5-trichloronitrobenzene is shown in Table 5. There was no significant change in the GSH content of kidney and the decrease in small intestine was barely significant. When rats were given butyl chloral hydrate (65 μmoles/100 g.) the GSH values for these tissues 1 hr. after dosage were not significantly lower than those for undosed rats. Expressed as in Table 5 they were 49 (20–91)[6] for kidney and 44 (36–49)[6] mg./100 g. for intestine.

Blood. The levels of glutathione observed in rat blood are recorded in Table 6. A significant and rapid decrease in glutathione content was found in rats dosed with butyl chloral hydrate, but the decrease in those dosed with 3:4:5-trichloronitrobenzene was not significant.

The GSH levels in the blood of three rabbits dosed with 3:4:5-trichloronitrobenzene are given in Table 7. There was a slight decrease only in rabbit 2. In the other two and in a rabbit given water only, the level is approximately constant over 5 hr. The administration of a large dose of benzyl chloride to rabbit 6 caused no significant decrease in the total glutathione of the blood (Table 7).

When glutathione was added to the blood of a rat by continuous injection no significant difference in the rate of excretion of mercapturic acid was observed in several experiments. The results of a typical experiment are summarized in Fig. 2. For these experiments 3:4-dichloronitrobenzene was used because the excretion of mercapturic acid from this compound is relatively slow (see Table 3 and Fig. 1) and there was adequate time for establishing the rate of excretion before the injection of glutathione was started.

Table 5. *Effect of* 3:4:5-*trichloronitrobenzene on levels of glutathione in rat kidney and intestine*

3:4:5-Trichloronitrobenzene (110 μmoles/100 g. of rat) was administered to rats which had fasted for 19 hr. Results are expressed as means with ranges in parentheses; the superior figures indicate the number of experiments. Values for 0 hr. are for undosed rats.

Time after dosage (hr.)	GSH (mg./100 g. of tissue)		
	Kidney	Small intestine	P for intestine
0	46 (23–65)[6]	46 (39–54)[6]*	—
0·5	40 (31–52)[6]	38 (31–46)[6]	0·05
1·0	—	38 (29–46)[6]	0·02
2·0	54 (45–78)[6]	42 (38–47)[6]	0·2
4·0	34 (25–56)[6]	37 (25–44)[6]	0·05
6·0	39 (27–55)[6]	40 (33–50)[6]	0·1
18·0	47 (39–55)[3]	52 (45–58)[3]	0·2

* Values for undosed animals which had fasted for 22 hr. and 25 hr. were 46 (40–55)[3] and 43 (38–47)[3] respectively.

Table 6. *Effect of* 3:4:5-*trichloronitrobenzene and butyl chloral hydrate on levels of glutathione in rat blood*

Rats were killed at times indicated. Results are expressed as means with ranges in parentheses; superior figures indicate the number of rats used. Values for 0 hr. are for undosed control rats.

Compound administered ...	3:4:5-Trichloronitrobenzene		Butyl chloral hydrate	
Dose (μmoles/100 g.) ...	110		65	
Time after dosage (hr.)	GSH (mg./100 ml.)	P	GSH (mg./100 ml.)	P
0	49 (38–65)[12]	—	49 (38–65)[12]	—
0·5	43 (42–46)[3]	0·2	34 (17–47)[5]	0·01
1·0	—	—	27 (21–35)[6]	<0·01
2·0	43 (41–45)[6]	0·1	25 (8–33)[4]	<0·02
4·0	43 (40–44)[3]	0·2	—	—
6·0	48 (40–60)[3]	0·8	—	—

Table 7. *Effect of mercapturic acid precursors on levels of glutathione in the blood of rabbits*

Doses were given to rabbits from which food had been withheld for 18 hr. Blood samples were withdrawn at the times indicated after dosage. Values for 0 hr. are for blood taken immediately before administration of the dose.

	Water	3:4:5-Trichloronitrobenzene (88 μmoles/100 g.)	Water		Benzyl chloride (250 μmoles/100 g.)	
	GSH	GSH	GSH	GSSG	GSH	GSSG
Rabbit ...	1	2, 3, 4	5	5	6	6
Time after dosage (hr.)						
0	60	49, 42, 42	35	8	16	14
0·25	61	54, 51, 44	—	—	—	—
0·5	58	45, 47, 42	26	11	23	10
1·0	—	42, 44, 42	—	—	—	—
1·5	54	40, 46, 42	—	—	—	—
1·75	—	— — —	25	19	17	13
2·0	—	39, 46, 42	—	—	—	—
3·0	59	38, 42, 42	—	—	—	—
3·75	—	— — —	30	9	24	18
4·0	54	37, 40, 42	—	—	—	—
5·0	58	36, 40, 43	—	—	—	—
5·5	—	— — —	34	6	24	10
6·75	—	— — —	32	11	25	9

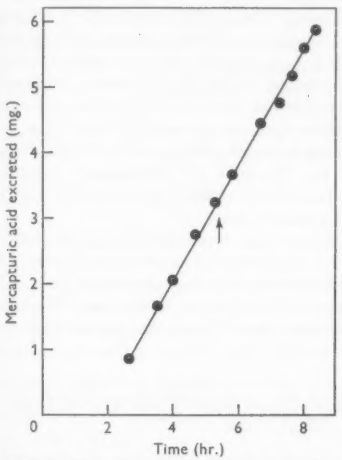

Fig. 2. Effect of injection of glutathione upon the rate of excretion of mercapturic acid by the rat. The rat was given 75 mg. of 3:4-dichloronitrobenzene in 3 ml. of water by stomach tube. Isotonic sodium chloride solution was then injected continuously into the caudal vein. At the time indicated by the arrow the injection fluid was changed to isotonic sodium chloride solution containing 1 mg. of GSH/ml. The injection rate was 3·3 ml./hr. over the period shown. The GSH equivalent to the mercapturic acid formed is 0·87 mg./hr.

DISCUSSION

It is clear from Table 2 that the rat can form mercapturic acids in effect by acetylcysteyldehalogenation and acetylcysteyldenitration from either aromatic precursors (types II*a* and III*a*) or aliphatic ones (types II*b* and III*b*) in amounts similar to those formed by the rabbit. The most conspicuous difference is that the rat excretes much less mercapturic acid from 3:4-dichloronitrobenzene than does the rabbit. For some reason rats seem to excrete this mercapturic acid relatively slowly (see Fig. 1).

No significant decrease in the GSH levels in blood was detected after the administration of 3:4:5-trichloronitrobenzene to rats or rabbits, nor was there any significant change in either GSH or GSSG levels in blood after the administration of benzyl chloride to rabbits. Furthermore, when glutathione was injected into the blood stream of the rat there was no detectable increase in the rate of excretion of mercapturic acid from 3:4-dichloronitrobenzene. The GSH content of rat blood was, however, considerably decreased after the administration of butyl chloral hydrate, which is not a mercapturic acid precursor. No change in the GSH level was detected in the kidneys of rats dosed with 3:4:5-trichloronitrobenzene, and in the intestine there was a slight decrease of doubtful significance. The intestine was examined because Anderson & Mosher (1951) found that a rapid synthesis of glutathione occurred in the intestinal mucosa of rats and it seemed possible that the mercapturic acid precursor would encounter this glutathione and react with it. The results in Table 5 suggest that if such a reaction occurs it can do so to only a very small extent.

Table 4 shows that when mercapturic acid precursors of types I, II and III are administered there is a fall in the level of liver glutathione which is most marked with benzyl chloride. As in general

there was no significant increase in GSSG the observed fall seems to be a true reduction in the concentration of GSH caused by the administered compound. This fall may be the result either of a direct combination of glutathione with the mercapturic acid precursor or of a secondary effect following the depletion of the cysteine in the animal as the mercapturic acid is formed. Binet & Wellers (1951) gave bromobenzene to rats and observed a slight rise in the level of total glutathione in the liver during the first hour after dosage and then a fall to about 40% of the initial level which persisted for about 48 hr. There followed on the third day a recovery to a level about 160% of the initial one and this gradually declined over nine days towards the initial level. They obtained similar results with kidney. The dose given by Binet & Wellers was 1900 μmoles/100 g. subcutaneously to 150 g. well-fed rats, whereas our dosage of chlorobenzene was 169 μmoles/100 g. orally to 200 g. fasting rats. Our dose caused a fall in the level of total glutathione to 15% of the initial value. The dose given by Binet & Wellers was so large as to produce serious toxic effects since they state that after 6–10 days 'la mortalité...devient sensible'. At our dose levels the animals showed no toxic symptoms and there was no mortality amongst those used only for collection of urine. The most conspicuous difference in results, apart from the more prolonged effect of the dose given by Binet & Wellers, was that in our experiments no significant change in the level of glutathione in the kidney was observed. In general our experiments were not continued beyond 6 hr. after dosage, but with 3:4:5-trichloronitrobenzene we did note that 18 hr. after dosage the GSH level in liver was higher than the initial level (see Table 4).

Simkin & White (1957) have shown that the turnover rate for free glycine in liver can be calculated from the depletion of free glycine in the liver and the rate of excretion of hippuric acid after the administration of the hippuric acid precursor, benzoic acid, by the formula $\mathbf{v} = v_h g_1/(g_1 - g_2)$, where $\mathbf{v}$ is the turnover rate, v_h the constant rate of formation of hippuric acid and g_1 and g_2 the steady free-glycine levels in the liver before and after the dose. If the liver glutathione is the immediate source of cysteine for mercapturic acid formation, then a similar formula, $\mathbf{v} = v_m g_1/(g_1 - g_2)$, where v_m is the constant rate of formation of mercapturic acid and g_1 and g_2 are the steady glutathione levels in the liver before and after the dose, would be expected to yield results for the turnover rate for glutathione in the liver comparable with the value found by other methods. For calculation of the turnover rates given in Table 8 the lowest values of Table 4 were used for g_2 and the corresponding control values for g_1. At these times the rate of excretion of the mercapturic acid was maximum and constant (Fig. 1). The mean turnover rates for GSH and for GSH+GSSG were 42 and 43 mg./100 g. of liver/hr. respectively (= 4·4 and 4·5 mg. of S/100 g./hr.).

The turnover rate for GSH in rabbit and rat liver was investigated by Waelsch & Rittenberg (1942) with [^{15}N]glutamic acid. They calculated a half-life period of 2–4 hr. Anderson & Mosher (1951) estimated that maximum incorporation of ^{35}S from [^{35}S]cystine into liver GSH in the rat took place in less than 3 hr. From the formula $t^{\frac{1}{2}} = 0{\cdot}7\ g/\mathbf{v}$ the turnover rates for GSH from Table 5 correspond with half-life periods of 2·1–4·0 (mean 3·1) hr. and those for GSH+GSSG with 2·0–3·8 (mean 3·1) hr., which are in good agreement with the results of Waelsch & Rittenberg (1942). For these calculations g, the normal level of glutathione, has been taken as 176 mg./100 g. of liver, the mean of the control values of Table 4. Although the values obtained for turnover rates cannot be regarded as precise they provide, in the absence of more precise data, a reasonable basis for the conclusion that the turnover rate for glutathione in liver would be sufficient to sustain the synthesis of the mercapturic acids. It should, however, be mentioned that Henriques *et al.* (1955) estimated that the half-life for glutathione in the rabbit was 7–9 hr., indicating a much slower turnover rate than that given by Waelsch & Rittenberg (1942), but Henriques *et al.* (1955) did not investigate the turnover rate in rats.

Butyl chloral hydrate did not significantly affect the level of glutathione in liver but caused a considerable fall in the level of glutathione in blood. The effect of butyl chloral hydrate therefore does not resemble that of a mercapturic acid precursor.

Table 8. *Turnover rates for glutathione in rat liver calculated from falls in level during formation of mercapturic acids*

The turnover rates $\mathbf{v}$ have been calculated from the formula $\mathbf{v} = v_m g_1/(g_1 - g_2)$. Values used for v_m are from Table 3, those for g_2 are the lowest values from Table 4 and those for g_1 those for the controls (water only) corresponding to the time period of the value taken for g_2. It has been assumed that the liver is 4% of the body weight.

Precursor	Turnover rate (mg./100 g. of liver/hr.) calculated from GSH	Turnover rate (mg./100 g. of liver/hr.) calculated from GSH+GSSG
Chlorobenzene	32	32
Benzyl chloride	49	50
p-Chlorobenzyl chloride	45	—
1-Bromobutane	59	63
3:4-Dichloronitrobenzene	31	33
3:4:5-Trichloronitrobenzene	42	—
2:3:4:6-Tetrachloronitrobenzene	34	36

Benzyl alcohol, however, caused a significant fall in the liver glutathione in 4 hr., with a recovery to the normal level in 6 hr. This may be due to the utilization of glycine for conjugation with the benzoic acid formed by oxidation from the alcohol, since almost the whole of a dose of benzyl alcohol is excreted by the rat as hippuric acid (unpublished result). 2:4:6-Trichloronitrobenzene, which forms neither a mercapturic acid to any appreciable extent nor a glycine conjugate, does not affect the level of glutathione in the liver. The results in this paper suggest that, when the mercapturic acids are formed from precursors, there is a fall in the level of glutathione in the liver, but not in blood, kidney or intestine, which is roughly commensurate with the amount of mercapturic acid formed. This suggests that an early stage in the formation of mercapturic acid might be the combination of the precursor with glutathione in the liver. The possibility that the mercapturic acid precursor combines with some other cysteine-containing compound, which breaks down to form the substituted cysteine and is itself reconstituted at the expense of cysteine from glutathione, has not been excluded.

SUMMARY

1. The possibility that glutathione may be involved in the formation of mercapturic acids *in vivo* has been examined.

2. The levels of glutathione in the liver fell when mercapturic acid precursors were given to rats; this fall was roughly commensurate with the amount of mercapturic acid formed.

3. No decrease in the glutathione levels in blood, kidney and intestine were observed in rats given mercapturic acid precursors. There was no significant change in rabbit blood under the same conditions.

4. The injection of glutathione into the caudal vein of the rat did not affect the rate of excretion of mercapturic acid.

5. Values are given for the amounts of mercapturic acid excreted by rats given various mercapturic acid precursors.

6. Turnover rates for liver glutathione calculated from the maximum rates of excretion of mercapturic acids and the levels of liver glutathione before and after administration of precursors are in agreement with those reported by other workers.

7. Butyl chloral hydrate, which is not a mercapturic acid precursor, caused no fall in the level of glutathione in liver but a pronounced fall in the level in blood.

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The Formation of Mercapturic Acids

2. THE POSSIBLE ROLE OF GLUTATHIONASE

By H. G. BRAY, T. J. FRANKLIN AND SYBIL P. JAMES
Physiology Department, The Medical School, University of Birmingham

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Barnes, James & Wood (1959) reported results which could be explained by the hypothesis that the first stage in the overall process of the formation of mercapturic acid from a precursor *in vivo* is the formation of an *S*-substituted glutathione from glutathione in the liver. If such a compound is an intermediate in the formation of mercapturic acid, it would be expected that enzymes of the liver would convert it into mercapturic acid. The effect of liver preparations on a conjugate of this type has, therefore, been examined. The compound chosen for study was *S*-(*p*-chlorobenzyl)glutathione, which may be an intermediate in the formation of *N*-acetyl-*S*-(*p*-chlorobenzyl)-L-cysteine from *p*-chlorobenzyl chloride *in vivo*. The percentage of a dose of *p*-chlorobenzyl chloride converted into this mercapturic acid in the rabbit is 23 (Bray, James & Thorpe, 1958*b*) and in the rat 37 (Barnes *et al.* 1959); so that livers of these animals would be expected to contain the necessary enzymes in sufficient amounts. The subsequent stages envisaged were the breakdown of the *S*-(*p*-chlorobenzyl)glutathione to yield *S*-(*p*-chlorobenzyl)-cysteine and the acetylation of *S*-(*p*-chlorobenzyl)-cysteine to yield *N*-acetyl-*S*-(*p*-chlorobenzyl)-L-cysteine. This paper is concerned with the breakdown of the glutathione complex. This might be achieved either by a hydrolytic reaction, e.g.

S-(*p*-chlorobenzyl)glutathione → glutamic acid + *S*-(*p*-chlorobenzyl)cysteine + glycine (1)

or by a transfer reaction, e.g.

S-(*p*-chlorobenzyl)glutathione + cysteine → glutathione + *S*-(*p*-chlorobenzyl)cysteine (2)

If the mechanism is a hydrolytic one, it might be catalysed by glutathionase, which is known to occur in liver (Neubeck & Smythe, 1944; Johnston & Bloch, 1951). A similar suggestion has been made by Binet & Wellers (1951).

EXPERIMENTAL

Materials and Methods

All melting points are uncorrected. Elementary microanalyses were carried out by F. and E. Pascher, Bonn.

Chlorobenzene, benzyl chloride, *p*-chlorobenzyl chloride, 1-bromobutane, 3:4-dichloronitrobenzene and 2:3:5:6-tetrachloronitrobenzene were purchased. 2:3:4:6-Tetrachloronitrobenzene was prepared as described by Betts, James & Thorpe (1955).

S-(p-*Chlorobenzyl)glutathione*. This was prepared from glutathione (cf. Stekol, 1941). A solution of glutathione (Boehringer und Söhne, G.m.b.H., Mannheim) (0·43 g. in 100 ml. of water) was made just alkaline to litmus with 2·5N-NaOH. Then 5 ml. of 2·5N-NaOH and 2 ml. of an ethanolic solution of *p*-chlorobenzyl chloride (0·8%) were added. Alternate additions of alkali and chloride solution (2 ml. of each) were made, with shaking, until 20 ml. more of each had been added over 15 min. Excess of chloride was removed by extraction of the reaction mixture with ether and the aqueous phase was acidified to pH 6·0 with 2N-HCl and left at 0° overnight. The granular precipitate was filtered off and the filtrate was concentrated at 50°

under reduced pressure to yield more solid material. The two solid fractions were combined and recrystallized from water. This gave 250 mg. of *S*-(*p*-chlorobenzyl)glutathione as a white amorphous powder which darkened progressively without melting on heating to 200° (Found: C, 47·5; H, 5·7; N, 9·3; Cl, 8·0; S, 7·6. $C_{17}H_{22}O_6N_3ClS$ requires C, 47·3; H, 5·1; N, 9·7; Cl, 8·2; S, 7·4%). After hydrolysis for 12 hr. with 6N-HCl at 100° it yielded *S*-(*p*-chlorobenzyl)-L-cysteine, glutamic acid and glycine, which were identified by paper chromatography as described below. *S*-(*p*-Chlorobenzyl)glutathione was also obtained from concentrated solutions of glutathione prepared from baker's yeast by the method of Schroeder, Collier & Woodward (1939). The yield was about 0·5 g. of *S*-(*p*-chlorobenzyl)glutathione from 2 lb. of yeast.

S-(p-*Chlorophenyl)glutathione.* This was prepared from diazotized *p*-chloroaniline and the cuprous mercaptide of glutathione by a method similar to that of Parke & Williams (1951) for phenylcysteines, except that the reaction mixture was kept overnight in the refrigerator. From 1 g. of glutathione 325 mg. of *S*-(*p*-chlorophenyl)-glutathione, m.p. 218° (decomp.), was obtained as a white amorphous powder (Found: C, 46·6; H, 5·3; N, 9·7; Cl, 8·4; S, 7·6. $C_{16}H_{20}O_6N_3ClS$ requires C, 46·0; H, 4·8; N, 10·0; Cl, 8·5; S, 7·7%).

S-(*p*-Chlorobenzyl)-L-cysteine, m.p. 202°, was prepared by a modification (Bray *et al.* 1958*b*) of the method of Suter (1895). *S*-(*p*-Chlorophenyl)-L-cysteine, m.p. 193°, was prepared by the method of Parke & Williams (1951).

Buffers. The following buffers were used. The pH was checked on a Cambridge pH meter with glass electrode. *For tissue slices.* Ringer–phosphate solution, pH 7·4, was prepared according to Umbreit, Burris & Stauffer (1949). *For homogenates.* 0·1M-Sodium phosphate buffers, pH 7·0 and pH 8·0, were as described by Britton (1932); sodium phosphate buffer with KCl + $MgSO_4$ was prepared according to Johnston & Bloch (1951), except that the sodium phosphate buffer used had pH 7·0 or 8·0 instead of 7·4.

Animals and tissues. Rats (about 200 g.), rabbits (about 2·5 kg.) and guinea pigs (about 500 g.) were killed by a sharp blow on the back of the head. The tissues were quickly removed and rapidly cooled in crushed ice. Slices (0·2–0·4 mm. thick) were cut by hand and immediately transferred to ice-cold Ringer–phosphate solution. Homogenates were prepared from 0·5 g. of tissue and 1 ml. of ice-cold buffer in a Potter–Elvehjem-type homogenizer with a Teflon pestle. The mixture was centrifuged at 2000 *g* for about 2 min. to remove larger particles and facilitate pipetting. The supernatant (homogenate) was added to an equal volume of a solution of the substrate in the chosen buffer in 100 ml. conical flasks fitted with a rubber bung with inlet and outlet tubes. The mixture was 'gassed' and shaken in a Warburg tank at 37° in an atmosphere of $O_2 + CO_2$ (19:1) or N_2 as appropriate.

The guinea pigs (about 500 g.) used for studying the excretion of mercapturic acid were maintained on a diet of rabbit pellets and water *ad lib.* during the experimental period. The compounds were administered with water by stomach tube. No toxic effects were observed except that 3:4-dichloronitrobenzene caused anorexia.

Glutathionase preparation. This was prepared from hog kidney which was frozen as soon as possible after the death of the animal and treated as described by Binkley & Olson (1951). Contrary to the experience of these workers it was found that reprecipitation of the enzyme reduced its activity. The preparation was, therefore, dried after the first precipitation with sodium acetate buffer (pH 4·0).

Determination of metabolites

Reduced glutathione. The method of Bhattacharya, Robson & Stewart (1955), depending on the activation of yeast glyoxalase by glutathione, was used.

Free glycine. The modification of Christensen, Riggs & Ray (1951) of the ninhydrin method of Alexander, Landwehr & Seligman (1945) was used.

Mercapturic acid in urine. *N*-Acetyl-*S*-(2-chloro-4-nitro)phenyl-L-cysteine was determined by the colorimetric method of Bray, James & Thorpe (1956). *N*-Acetyl-*S*-benzyl-L-cysteine, *N*-acetyl-*S*-(*p*-chlorobenzyl)-L-cysteine and *N*-acetyl-*S*-butyl-L-cysteine were determined by the nitroprusside method described by Bray *et al.* (1958*b*). *N*-Acetyl-*S*-(*p*-chlorophenyl)-L-cysteine, *N*-acetyl-*S*-(2:3:4:6-tetrachlorophenyl)-L-cysteine and *N*-acetyl-*S*-(2:3:5:6-tetrachlorophenyl)-L-cysteine were determined by the modification (Betts *et al.* 1955) of the iodometric titration method of Stekol (1936).

Determination of glutathionase activity of small intestine

The small intestine was excised from a rat immediately after death, and its contents were removed. The intestine was cut into small pieces and ground with sand in 25 ml. of phosphate buffer (pH 8·0). The mixture was then centrifuged for 15 min. at 2000 *g*. For assay of glutathionase, the supernatant (5 ml.) and substrate (30 μmoles in 5 ml. of water) were incubated at 37° with shaking for 4 hr. Then 10% (w/v) $ZnSO_4,7H_2O$ (5 ml.) and 0·5N-NaOH (5 ml.) were added. The mixture was filtered and the filtrate was diluted tenfold with water for determination of glycine.

Paper chromatography

Centrifuged homogenates were applied to the paper. Phenol red, phenylalanine and tyrosine were used as markers with the monophasic solvent mixture given in Table 1, which gives for the compounds examined the rates of flow, R_P, relative to phenol red. In addition to R_P values, *S*-(*p*-chlorobenzyl)glutathione and *S*-(*p*-chlorobenzyl)-L-cysteine were readily distinguished by the different colours given with the ninhydrin reagent. The corresponding *p*-chlorophenyl compounds could not be so distinguished. The approximate amounts of *S*-(*p*-chlorobenzyl)-L-cysteine and *S*-(*p*-chlorophenyl)-L-cysteine liberated were estimated by comparison of the areas and intensities of the spots obtained. The extracts were applied from an Agla syringe. The minimum amount of each compound which could be detected was about 5 μg.

RESULTS

Liberation of S-(p-*chlorobenzyl*)-L-*cysteine from* S-(p-*chlorobenzyl*)*glutathione*

In liver slices. Liver slices were incubated with 0·06M-*S*-(*p*-chlorobenzyl)glutathione in Ringer–phosphate buffer (pH 7·4) for 4 hr. at 37° with gas phase $O_2 + CO_2$ (19:1). After incubation the

medium, or a homogenate of the slices in the medium, was examined for *S*-(*p*-chlorobenzyl)-cysteine by paper chromatography. This compound was detected in the reaction mixture from slices of guinea-pig liver but not in those from rat liver. As shown below, rat liver is less active than guinea-pig liver in liberating *p*-chlorobenzylcysteine from the *p*-chlorobenzylglutathione.

In homogenates of rat liver. Rat-liver homogenates were incubated with *S*-(*p*-chlorobenzyl)-glutathione in buffer containing adenosine triphosphate (ATP) and L-cysteine. The results are shown in Table 2. Chromatographic analysis of the supernatant from the centrifuged digests showed the presence of *S*-(*p*-chlorobenzyl)-L-cysteine. Boiled homogenates gave negative results. If the mechanism of the process involves the transfer of the *p*-chlorobenzyl residue from the glutathione complex to cysteine, glutathione should be formed as the reaction proceeds (see equation 2). The digests contained L-cysteine as a possible acceptor for the *p*-chlorobenzyl residue in such a reaction. In two homogenates a 90–100% fall in the level of glutathione occurred in both controls and experiments. While these results did not indicate a net formation of glutathione, there might have been formed a small amount of glutathione, which was masked by a relatively large loss due to other reactions, e.g. hydrolysis or oxidation. Attempts were made to eliminate the effect of oxidation by using a gaseous phase of pure nitrogen, but a considerable disappearance of glutathione still occurred in the absence of *S*-(*p*-chlorobenzyl)glutathione; this might have been due to hydrolysis by a glutathionase system similar to that described by Binkley & Olson (1951) and Johnston & Bloch (1951). Experiments were carried out with a medium containing, in addition to the usual constituents, glutathione, which is reported by

Table 1. *Paper chromatography of* S-(p-*chlorobenzyl*)-L-*glutathione and reference compounds*

The descending method was used with phenol red as marker. The solvent mixture was *n*-butanol–water–acetic acid–butyl acetate (24:10:5:2, by vol.) and the detecting reagent 0·1% ninhydrin in acetone containing 1 drop of collidine/100 ml. (Woiwod, 1949). The time of run was 16 hr., by which time the solvent front had moved off the paper. Rates of flow, R_P, are expressed relative to that of phenol red taken as 1·0.

Compound	R_P	Colour of detected spot
Glycine	0·30	Purple
L-Glutamic acid	0·43	Purple
L-Tyrosine	0·73	Purple
DL-Phenylalanine	0·87	Purple
Phenol red	1·0	Yellow
S-(*p*-Chlorobenzyl)glutathione	1·12	Purple
S-(*p*-Chlorobenzyl)-L-cysteine	1·31	Blue
S-(*p*-Chlorophenyl)glutathione	1·05	Purple
S-(*p*-Chlorophenyl)cysteine	1·22	Purple
Glutathione,* GSH	0·21	Purple
GSSG	0·07	Purple
	0·36	Purple

* Glutathione gives a purple colour with ninhydrin reagent when about 100 μg. is applied to the paper. (The other compounds listed above can be detected in amounts of about 5 μg.) The GSH used gave a faint spot (probably GSSG), R_P 0·07. The main spot from GSSG had R_P 0·07. The GSH spot (R_P 0·21) first appeared as a purple ring with a colourless centre, but after several hours the whole spot became purple.

Table 2. *Changes in glutathione levels and liberation of* S-(p-*chlorobenzyl*)-L-*cysteine from* S-(p-*chlorobenzyl*)*glutathione*

Medium: sodium phosphate buffer (pH 8·0 unless stated otherwise) with KCl and $MgSO_4$ to which was added, for each millilitre, L-cysteine hydrochloride (2·5 mg.), ATP (2·5 mg.) and, where indicated, *S*-(*p*-chlorobenzyl)glutathione (CBG) (2·5 mg.) or glutathione (GSH) (0·5 mg.). The homogenate was mixed with an equal volume of medium and incubated at 37° with continuous shaking. Values are means of duplicate determinations in close agreement.

Additions	Time of incubation (hr.)	Percentage fall in level of GSH	Relative intensity of *S*-(*p*-chlorobenzyl)-L-cysteine spot on chromatogram
	Gas phase: O_2+CO_2 (19:1)		
None	4	100, 90	—
CBG	4	90, 90	+ +, + +
	Gas phase: N_2		
None	4	80	—
CBG	4	85	+ +
CBG (pH 7·0)	4	75	+
CBG + GSH	4	20	Trace
CBG + GSH (pH 7·0)	4	18	–
None	2	40, 40	—
GSH	2	35, 25	—
GSH + CBG	2	27, 24	–, –
GSH (pH 7·0)	2	16, 12	—
GSH + CBG (pH 7·0)	2	16, 14	–, –

Johnston & Bloch (1951) to stimulate the glutathione-synthesizing systems in pigeon-liver homogenates and by Binkley & Olson (1951) to inhibit glutathionase in hog kidney. The reaction was studied also at pH 7·0, at which, according to Binkley & Olson (1951), the activity of the glutathionase system is diminished. Evidence of glutathione synthesis was not obtained, so that a transfer mechanism seemed unlikely. In experiments where the fall in the level of glutathione was diminished significantly, the release of *S*-(*p*-chlorobenzyl)-L-cysteine was also diminished and was often negligible. The addition of glutathione to the digests and the use of pH 7·0 diminished the fall in the level of glutathione. It seemed, therefore, likely that the disappearance of glutathione could be attributed to the action of glutathionase and that the appearance of *S*(*p*-chlorobenzyl)-L-cysteine was the result of a hydrolytic process.

In liver homogenates of guinea pig and rabbit. The results are given in Table 3. Advantage was taken of the fact that glutathionase is inhibited by phenol red and bromosulphalein (Binkley & Olson, 1951). The results show that these two compounds inhibit both the fall in the level of glutathione and the release of *S*-(*p*-chlorobenzyl)-L-cysteine in the preparations used. This observation provides further evidence that glutathionase may be involved in the formation of *S*-(*p*-chlorobenzyl)-L-cysteine from *S*-(*p*-chlorobenzyl)glutathione.

Table 3. *Effect of glutathionase inhibitors on liberation of* S-(p-*chlorobenzyl*)-L-*cysteine from* S-(p-*chlorobenzyl*)*glutathione and on changes in glutathione levels in guinea-pig and rabbit-liver homogenates*

Medium: 0·1 M-sodium phosphate buffer (pH 8·0) to which was added, for each millilitre, *S*-(*p*-chlorobenzyl)-glutathione (2·5 mg.) and, where indicated, glutathione (GSH) (0·5 mg.), phenol red (2 mg.) or bromosulphalein (2 mg.). The homogenate was mixed with an equal volume of medium and incubated with continuous shaking at 37° for 2 hr. Gas phase: N_2. Values are means of duplicate determinations.

Additions	Percentage fall in glutathione level	Intensity of *S*-(*p*-chlorobenzyl)-L-cysteine spot
	Guinea pig	
None	96	+ + +
GSH	96*	+ + +
Phenol red	26	+
Bromosulphalein	45	+
	Rabbit	
None	77	+ +
GSH	43	+
Phenol red	64	−
Bromosulphalein	69	−

* Guinea-pig liver is a richer source of glutathionase than is rabbit liver and this result presumably indicates that the amount of GSH added in this experiment was insufficient to inhibit the action of glutathionase.

Liberation of glycine from S-(p-*chlorobenzyl*)-*glutathione by liver homogenates*

Although paper chromatography gave satisfactory comparative estimates of the amounts of *S*-(*p*-chlorobenzyl)-L-cysteine liberated, a more accurate estimate of the breakdown of glutathione was desirable. If the formation of *S*-(*p*-chlorobenzyl)-L-cysteine involves complete hydrolysis of the tripeptide, glutamic acid and glycine should be liberated at the same time. According to Fodor, Miller & Waelsch (1953), free glutamic acid is not produced in homogenates, but is transferred to acceptors, e.g. other amino acids. Free glycine is released, however, and this can be readily determined quantitatively. The results obtained (Table 4) show that, with all three tissues, the conditions under which additional glycine is liberated are similar to those in which the liberation of *S*-(*p*-chlorobenzyl)-L-cysteine occurs. The relatively high control values are presumably due to the hydrolysis of glutathione present normally in the tissues. The inhibitory effect of phenol red again suggests the participation of glutathionase.

Two experiments were carried out with homogenates of guinea-pig liver and *S*-(*p*-chlorophenyl)-glutathione. Under the conditions described in

Table 4. *Effect of phenol red on liberation of glycine from* S-(p-*chlorobenzyl*)*glutathione by liver homogenates*

Medium: 0·1 M-sodium phosphate buffer (pH 8·0) to which was added, where indicated, for each millilitre, *S*-(*p*-chlorobenzyl)glutathione (CBG) (2·5 mg.) and phenol red (2 mg.). The homogenate was mixed with an equal volume of medium and incubated at 37° with continuous shaking for 4 hr. Gas phase: N_2. Values are means of duplicate determinations. Initial concentrations of glycine were 166–225 μg./ml. for guinea-pig liver, 48–62 for rat liver and 48 for rabbit liver.

Additions	Increase in glycine concentration (μg./ml.) over initial concentration		
	Guinea pig	Rat	Rabbit
None (control)	145, 111, 97	76, 39	65
CBG	262, 362, 270	153, 72	100
Phenol red	—, —, 67	—, 0	20
CBG + phenol red	—, —, 169	—, 5	40

Table 4 the control values were 72 and 67 and the values when *S*-(*p*-chlorophenyl)glutathione was added were 388 and 266 μg. of glycine/ml. respectively.

Glutathionase activity of small intestine

The extent of hydrolysis of glutathione and of *S*-(*p*-chlorobenzyl)glutathione by extracts of rat small intestine was determined from the glycine liberated. The results, expressed as percentage of substrate hydrolysed under the conditions given in the Experimental section, were 60 and 55 % for glutathione and 65 and 60 % for *S*-(*p*-chlorobenzyl)-glutathione. (Each value is the mean of duplicate determinations.) Examination of digests from *S*-(*p*-chlorobenzyl)glutathione by paper chromatography revealed the presence of *S*-(*p*-chlorobenzyl)-L-cysteine.

Table 5. *Liberation of glycine from glutathione and from* S-(p-*chlorobenzyl*)*glutathione by a preparation of glutathionase*

Substrates consisted of 10 ml. of 0·1 M-sodium phosphate buffer (pH 8·0) containing either 25 mg. of *S*-(*p*-chlorobenzyl)glutathione or 17·8 mg. of glutathione. Phenol red (20 mg.) and glutamine (20 mg.) were added as indicated. Tubes were incubated with 15 mg. of glutathionase preparation at 37°. Duplicate 1 ml. samples were withdrawn at intervals for analysis and the mean results are given. There was no free glycine at the start of any experiment. In the experiments with *p*-chlorobenzylglutathione additional samples were withdrawn and centrifuged. Portions (30 μl.) of the supernatant were spotted on sheets for analysis by paper chromatography.

Additions to substrate	Glycine liberated (μg./ml. of digest) 1 hr.	2 hr.	4 hr.	Relative intensity of *p*-chlorobenzyl-cysteine spot
	Glutathione			
None	38	64	113	–
Phenol red	38	48	62	–
Glutamine	54	140	257	–
	S-(*p*-Chlorobenzyl)glutathione			
None	78	153	265	+ + +
Phenol red	48	86	172	+
Glutamine	161	262	359	+ + + +

Experiments with a glutathionase preparation

The ability of the glutathionase preparation to liberate *S*-(*p*-chlorobenzyl)-L-cysteine from *S*-(*p*-chlorobenzyl)glutathione was first established by paper chromatography. For quantitative experiments, however, it was more satisfactory to assess its activity in terms of the liberation of glycine. Typical results are shown in Table 5. The inhibition by phenol red of the liberation of glycine from both glutathione and *S*-(*p*-chlorobenzyl)glutathione again supports the hypothesis that glutathionase is involved in mercapturic acid formation. The enhancement of the hydrolytic activity of glutathionase by glutamine is also very marked (Table 5). This property of glutathionase was noted by Binkley & Olson (1951), but we could not demonstrate it in slice and homogenate preparations, possibly because of the presence of glutaminase. It is of interest that *S*-(*p*-chlorobenzyl)-glutathione was more readily hydrolysed than is glutathione itself.

Formation of S-(p-*chlorobenzyl*)*glutathione from* p-*chlorobenzyl chloride* in vivo

A rabbit was given 0·5 g. of *p*-chlorobenzyl chloride and was killed 2 hr. later. The liver was removed and homogenized. The supernatant from the centrifuged homogenate was examined by paper chromatography with *S*-(*p*-chlorobenzyl)-cysteine and *S*-(*p*-chlorobenzyl)glutathione as reference compounds. In addition to slow running material, spots of R_F 1·1 and 1·3 corresponding to the two reference compounds in position and colour were observed. This result was obtained in several experiments on paper sheets, but attempts to achieve unequivocal identification by separation of these compounds on a larger scale on columns have so far been unsuccessful.

Excretion of mercapturic acids by the guinea pig

The amounts of mercapturic acid excreted by the guinea pig after administration of precursors are given in Table 6. The results are much lower than those obtained from the rat and rabbit under similar conditions (see Barnes *et al.* 1959).

Table 6. *Excretion of mercapturic acid by the guinea pig*

Results are expressed as percentages of the dose, given as means with ranges in parentheses; superior figures indicate the number of experiments. Samples of urine were collected until mercapturic acid could no longer be detected (usually 24 hr.).

Compound administered	Dose (μmoles/100 g.)	Mercapturic acid excreted
Chlorobenzene	200	<1 (<1, <1)[2]
Benzyl chloride	185	4 (2–6)[4]
p-Chlorobenzyl chloride	135	5 (0–8)[3]
1-Bromobutane	108	19 (11–23)[5]
3:4-Dichloronitrobenzene	104	3 (2–5)[5]
2:3:4:6-Tetrachloronitrobenzene	96	2 (0–4)[3]
2:3:5:6-Tetrachloronitrobenzene	96	0[3]

DISCUSSION

Experiments of earlier workers on the possible role of glutathione in the formation of mercapturic acids have been briefly reviewed by Barnes *et al.* (1959). Stekol (1940, 1941) showed that oral administration of *S*-benzylglutathione and *S*-(*p*-bromobenzyl)glutathione to rats led to the excretion of *N*-acetyl-*S*-benzyl-L-cysteine and *N*-acetyl-*S*-(*p*-bromobenzyl)-L-cysteine respectively in urine. Our results with rat intestine suggest that glutathione and its derivatives are likely to be extensively hydrolysed in the intestine before absorption and thus cast doubt on the value of Stekol's observations as evidence for the participation of glutathione in mercapturic acid formation. The results reported in this and the preceding paper (Barnes *et al.* 1959) are compatible with the view that stages in the formation of mercapturic acids *in vivo* are the combination of the precursor with glutathione in the liver and subsequent hydrolysis of the glutathione complex by glutathionase to *S*-substituted cysteine, glycine and glutamic acid. The existing evidence does not preclude the possibility that conjugation of a precursor of mercapturic acid may occur with a precursor of glutathione rather than with glutathione itself. Such a reaction could account for the failure of intravenously administered glutathione to increase the amount or rate of excretion of mercapturic acid (Barnes *et al.* 1959). Whether or not a mechanism of mercapturic acid formation involving glutathione is likely to be the only mechanism, applying to all types (see Barnes *et al.* 1959) of precursors of mercapturic acids, cannot yet be decided.

The results in the present paper are of particular interest in connexion with species differences. Neubeck & Smythe (1944) found that guinea-pig liver was a richer source of glutathionase than was rabbit or rat liver and gave the relative activities as 100:17:2–5, the last being within the experimental error of the determination. Binkley & Nakamura (1948) found that rat-liver homogenates were inactive in this respect. Our results confirm the observation of Neubeck & Smythe (1944) that guinea-pig liver is the richest in glutathionase activity, but indicate that the activity of rat liver is of the same order as that of rabbit and is certainly not negligible. The activity of rabbit and rat liver was such as might have been expected if glutathionase were involved in the formation of mercapturic acids *in vivo*; both animals excrete these acids readily when given precursors. On the same basis it would have been expected that the guinea pig would be at least as well equipped to form mercapturic acids. From the results in Table 6, however, it is clear that this is not generally true; the guinea pig in fact appears to excrete barely significant amounts of mercapturic acid from most of the precursors examined. This abnormality of the guinea pig has been observed by Corner & Young (1954), who isolated only 0·6% of naphthylmercapturic acid from the urine of guinea pigs to which naphthalene had been given. From the urine of rabbits, rats and mice similarly dosed the yields of naphthylmercapturic acid were 4, 12 and 6% respectively (Bourne & Young, 1934; Young, 1947). A possible explanation of this species difference would be that the failure of the guinea pig to excrete appreciable amounts of mercapturic acid is due, not to failure of glutathionase to hydrolyse the *S*-substituted glutathione, but to failure to acetylate the *S*-substituted cysteine liberated (Bray, Franklin & James, 1958*a*).

SUMMARY

1. The effect of liver preparations and of glutathionase on *S*-(*p*-chlorobenzyl)glutathione has been studied.

2. *S*-(*p*-Chlorobenzyl)glutathione was hydrolysed to *S*-(*p*-chlorobenzyl)cysteine by slices of guinea-pig liver, by homogenates of guinea-pig, rat and rabbit liver and by a preparation of glutathionase from hog kidney.

3. Glycine was liberated simultaneously and its determination provided a convenient method for the quantitative study of the reaction.

4. The results obtained are compatible with the hypothesis that the hydrolysis of *S*-(*p*-chlorobenzyl)glutathione to *S*-(*p*-chlorobenzyl)cysteine is a stage in the formation of mercapturic acid from *p*-chlorobenzyl chloride *in vivo*.

5. *S*-(*p*-Chlorobenzyl)glutathione was readily hydrolysed by extracts of rat small intestine.

6. Although guinea-pig liver is richer than is rabbit or rat liver in glutathionase, the guinea pig does not in general form as large amounts of mercapturic acid from various mercapturic acid precursors as do the other two species.

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Hormonal Factors Affecting Glucose 6-Phosphatase Activity

1. EFFECT OF HYPOPHYSECTOMY AND REPLACEMENT THERAPY IN THE RAT

By A. E. HARPER* and F. G. YOUNG
Department of Biochemistry, University of Cambridge

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Cope & Marks (1934) observed that in hypophysectomized animals the liver glycogen appeared to be less responsive to glycogenolytic stimuli than that in normal animals. The present investigation was undertaken to ascertain whether in the hypophysectomized rat there is a diminution in liver glucose 6-phosphatase activity which might contribute to such a sluggishness in glycogenolysis, and further whether any diminution in glucose 6-phosphatase observed to follow removal of the pituitary gland in the rat could be prevented or cured by treatment with hormones.

METHODS

Animals. White rats of the Wistar strain, bred in the Laboratory, were used. Unless otherwise stated, they consumed a stock diet *ad lib.* to such an extent that their body weight rose by 35–40 g. each week. In some experiments pair-feeding was carried out. By this is meant that a pair of animals, one control and one experimental, chosen to be comparable in body weight, were fed the same amount of food each day. If one animal ate less than the other on any day, both were given the smaller amount the following day. In some experiments the principle of pair-feeding was extended to three or four comparable animals, the amount consumed by the animal eating least being fed to all.

Hypophysectomy was carried out by the parapharyngeal route. In any animal in which signs of incomplete hypophysectomy had been observed, such as incomplete cessation of growth or failure of the gonads to atrophy, completeness of removal of the gland was checked *post mortem* by visual examination of the sella turcica. If necessary the results for the animal were discarded. Alloxan diabetes was induced in intact rats by the intraperitoneal injection of 40 mg. of alloxan/kg. body wt. Only rats which exhibited frank glycosuria were used in the experiments. Some of the alloxan-diabetic rats were subsequently hypophysectomized.

Hormone preparations. Growth hormone was prepared in the Laboratory by a method essentially that of Wilhelmi, Fishman & Russell (1948). For injection it was dissolved in 0·9% NaCl soln. at a concentration of 5 mg./ml., and 0·2 ml. of this solution (1 mg. of hormone) was administered daily by the subcutaneous route to rats under treatment. Cortisol, suspended in 0·9% NaCl soln. at a concentration of 5 mg./ml., was administered subcutaneously at a rate of 1 mg./rat/day. Sodium-L-thyroxine was dissolved in 0·9% NaCl soln. by the addition of NaOH to give a concentration of 0·5 mg. of thyroxine/ml. Of this solution 0·2 ml. (equivalent to 0·1 mg.) was subcutaneously administered each day to a rat under treatment with this hormone.

Estimation of glucose 6-phosphatase. Glucose 6-phosphatase activity was measured in a tissue homogenate. The rat was stunned, decapitated and bled, and the needed organ removed and chilled in ice. A weighed sample of the organ (200–400 mg.) was homogenized in ice-cold 0·02M-acetate–borate–cacodylate buffer, pH 6·5 (Plumel, 1948; de Duve, Berthet, Hers & Dupret, 1949), in a Potter–Elvehjem glass homogenizer. Enzyme activity was determined by the method of de Duve *et al.* (1949) but with a final substrate concentration of 0·04M. The total volume of 0·6 ml. used

* Present address: Department of Biochemistry, University of Wisconsin, Madison 6, Wisconsin, U.S.A.

contained material from approx. 5 mg. of tissue. Phosphorus was estimated by the method of Fiske & Subbarow (1925). In agreement with Hers & de Duve (1950) significant hydrolysis of glucose 6-phosphate between pH 6 and pH 7 was found only with homogenates of liver, kidney and small intestine. The preparations from intestine were activated by Mg^{2+} ions and were optimally active at pH 9–10 so we have confined our recorded observations to liver and kidney preparations at pH 6·5.

The results of the estimation of glucose 6-phosphatase activity were expressed in two different ways. The first is in terms of mg. of P released/g. wet wt. of tissue/10 min. For convenience, results expressed in this way are referred to as 'enzyme concentration', without prejudice to the question of whether, when changes occurred, the amount of protein enzyme had varied or whether the activity of the enzyme/mg. of enzyme protein had altered. The second way in which results have been expressed is in terms of mg. of P calculated to be released by the tissues of the whole of an organ in 10 min., reduced to a figure for each 100 g. body wt. For convenience this figure is referred to as 'total activity/100 g. body wt.' for the organ in question. Where changes in glucose 6-phosphatase activity are referred to, without specific qualifications, the changes in values expressed in both ways are being considered. Both of these methods have disadvantages but there exists no simple single solution to the problem of the terms in which the enzyme content of tissues are best given. If the activity is expressed in terms of activity/g. wet wt. of tissue a fall in the amount of any of the major constituents, and particularly that of glycogen and water, could result in an apparent increase in enzyme activity, even though no rise in the total amount of enzyme in the tissue occurred. Similar considerations apply to expression in terms of activity/g. dry wt. of tissue, to activity/mg. of N and to activity/mg. of deoxyribonucleic acid P. Nevertheless results expressed in terms of total activity per organ should be uninfluenced by changes in the composition of the organ, and we have adopted the procedure of expressing total enzyme activity in an organ/100 g. body wt. to allow for both the changes in weight of the organ and the alterations in weight of the animal.

Blood sugar was estimated by the method of Somogyi (1952). Glycogen was determined by the method of Good, Kramer & Somogyi (1933), the sugar being estimated by the method of Somogyi (1952).

RESULTS

Effect of hypophysectomy on glucose 6-phosphatase activity

Liver. In control experiments (Fig. 1) the relationship between body weight and glucose 6-phosphatase activity of liver tissue was assessed for the intact growing rats of our colony. The changes in enzyme concentration in liver tissue are less striking than those in total activity/100 g. body wt. (Fig. 1). Both values rise as body weight increases from 50 to 100 g., and then decline, the difference in total activity/100 g. body wt. being substantial and highly significant ($P < 0·01$) between 100 and 350 g. rats.

Starvation for 24 hr. caused a substantial rise in glucose 6-phosphatase concentration in the livers of both the young and older rats (Fig. 1), the difference between the average values for the old and young animals being approximately the same after starvation as it was before.

When the pituitary gland was removed from the rat the glucose 6-phosphatase activity of the liver fell quite rapidly for about 2 weeks after the operation, and then more slowly or not at all (Fig. 2). The results given in Fig. 2 for enzyme

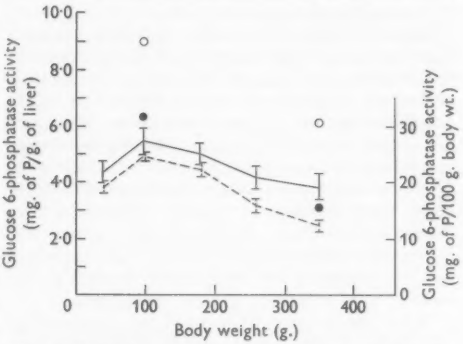

Fig. 1. Effect of age on the glucose 6-phosphatase activity of the liver of the normal (non-starving) rat. The lengths of the vertical lines on the points of the curves plotted indicate the standard errors of the mean values illustrated. ——, Activity/g. of liver; - - - -, total activity/100 g. body wt. Circles indicate the enzyme activities in the liver after starvation of rats of different ages for 24 hr.: ●, total activity/100 g. body wt.; ○, activity/g. of liver.

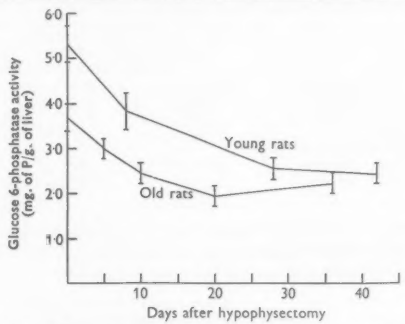

Fig. 2. Effect of hypophysectomy on the glucose 6-phosphatase concentration (mg. of P released/g. of wet tissue/10 min.) in the livers of young and of older rats. The upper curve gives the results for rats of 100–150 g. body wt. before hypophysectomy, and the lower one is for rats of 250–350 g. before hypophysectomy. The lengths of the vertical lines on the points of the curves plotted indicate the standard errors of the mean values illustrated.

concentration are similar to those, not illustrated, for total activity/100 g. body wt. and indicate that, despite the different initial values, the rate of fall is similar in older and in young rats.

In order to ascertain if the fall in activity after hypophysectomy resulted from loss of an activator of the enzyme, or possibly from the accumulation of a substance which depressed the activity of the enzyme, some experiments were carried out with mixtures of homogenates of liver tissue from normal and from hypophysectomized rats. No difference between the expected activity of the mixture and that actually observed was found.

Since the hyophysectomized rat eats less than a normal one, and starvation of an intact rat leads to a rise in liver glucose 6-phosphatase activity (Ashmore, Hastings, Nesbett & Renold, 1956), it was of interest to compare the enzyme activity in the livers of intact rats pair-fed with hypophysectomized ones, and of normal rats fed *ad lib.* with hypophysectomized animals. The results (Table 1) show that pair-feeding greatly emphasizes the difference in enzyme concentration between the livers of normal and of hypophysectomized rats but has less influence on comparisons made on the basis of total activity/100 g. body weight.

When the hypophysectomized rat was starved for 24 hr. a small but highly significant rise ($P<0.01$) in liver glucose 6-phosphatase concentration was seen (Table 2), though this was less than that seen for the starving intact rat (Ashmore, Hastings & Nesbett, 1954; Harper, 1959). The rise in total activity/100 g. original body wt. observed in these experiments (Table 2) was not significant ($P>0.1$). The feeding of protein (casein) *ad lib.* to the starving hypophysectomized rat (Table 2) led to a rise in the glucose 6-phosphatase activity of the liver that was just significant (P 0·05), despite the fact that the feeding of casein to the normal rat causes a highly significant rise in the total enzyme activity in the liver (Harper, 1959).

Table 1. *Comparison of liver glucose 6-phosphatase activity of hypophysectomized rats with that of intact controls fed* ad libitum *or pair-fed*

Condition of animal	Liver glucose 6-phosphatase activity (mean ± S.E.M.) (mg. of P/g. of liver)	(mg. of P/100 g. body wt.)
Control (hypophysectomized)	2·9 ± 0·5	8·8 ± 1·2
Normal: pair-fed	8·0 ± 0·7	26·7 ± 1·7
Normal: fed *ad lib.*	5·2 ± 0·6	22·8 ± 1·6

Table 2. *Effects of starvation and of feeding protein alone after starvation on the liver glucose 6-phosphatase activity of hypophysectomized rats*

Condition of animal	Liver glucose 6-phosphatase activity (mean ± S.E.M.) (mg. of P/g. of liver)	(mg. of P/100 g. body wt.)
Control (hypophysectomized)	2·2 ± 0·2	6·7 ± 0·5
Starved 24 hr.	3·6 ± 0·2	9·4 ± 0·4
Starved 24 hr. and then fed casein *ad lib.* for 24 hr.	3·8 ± 0·1	11·8 ± 0·8

Kidney. The glucose 6-phosphatase activity of the kidney of the hypophysectomized rat is not significantly ($P>0.1$) lower than that of the normal rat, though the mean values for hypophysectomized rats were a little below those for control intact rats. With normal rats typical mean values for enzyme concentration, expressed as mg. of P/g. of tissue/10 min., for liver and kidney were 4·84 ± 0·2 and 4·54 ± 0·3 respectively, whereas with comparable hypophysectomized rats the corresponding values were 2·80 ± 0·3 and 4·06 ± 0·3. In view of these findings no further investigations were carried out of hormonal effects on kidney glucose 6-phosphatase activity.

Influence of treatment with hormones on the liver glucose 6-phosphatase activity of the hypophysectomized rat

Growth hormone. Since treatment with growth hormone raises the food intake of the hypophysectomized rat, experiments both with paired-feeding and with feeding *ad lib.* were carried out. These experiments were performed in conjunction with those on cortisol (see below) and in general the food intake of hypophysectomized control rats and of hypophysectomized growth-hormone-treated rats was restricted to that of cortisol-treated hypophysectomized rats, the food intake of the last group being lowest. With feeding *ad lib.*, treatment with growth hormone for 2 weeks had little influence on the liver glucose 6-phosphatase activity of the hypophysectomized rat (Table 3). When the food intake of the growth-hormone-treated hypophysectomized rat was the same as that of the hypophysectomized rat, the total enzyme activity/100 g. body wt. was similar in the two groups ($P>0.1$), but the enzyme concentration in the livers of growth-hormone-treated hypophysectomized rats was significantly greater ($P<0.05$) than that in untreated hypophysectomized animals (Table 3).

Cortisol. When food intake was uncontrolled the administration of cortisol for 2 weeks to the hypophysectomized rat significantly raised both the enzyme concentration in the liver and total activity/100 g. body wt. ($P<0.05$) above the corresponding values for hypophysectomized rats (Table 3). When these groups were pair-fed the food intake of the cortisol-treated hypophysectomized rats was lowest, so that these animals were

in effect eating *ad lib.* in both experiments. This is the reason for the fact that the values for glucose 6-phosphatase activity for these animals are similar whether or not they were pair-fed. On the other hand, with pair-feeding control the other groups suffered some measure of food restriction and the values for the hypophysectomized control rats (Table 3) were higher, resembling those observed after starvation for 24 hr. (Table 2). Under these conditions there was no evidence of an effect of cortisol on glucose 6-phosphatase concentration, and total enzyme activity/100 g. body wt. was also not significantly different ($P > 0.1$) from that of the control animals (Table 3).

Growth hormone plus cortisol. When both of these hormones were given to the hypophysectomized rat for 2 weeks with feeding *ad lib.* (Table 3) both the enzyme concentration in the liver and the total enzyme activity/100 g. body wt. rose in a highly significant manner ($P < 0.01$). With pair-feeding control the total enzyme activity/100 g. body wt. was not significantly different ($P > 0.1$) from that of hypophysectomized control rats (Table 3), though the concentration of enzyme was significantly increased ($P < 0.01$).

Thyroxine plus cortisol. When both thyroxine and cortisol were administered to the hypophysectomized rat for 4 days the glucose 6-phosphatase activity in the liver was highly significantly increased ($P < 0.01$), the result being greater than would be expected if the effects of the two hormones were only additive (Table 4). After a single injection of both hormones, the liver glucose 6-phosphatase activity was no greater than it was when thyroxine only was administered (Table 5) although, as before, 4 days of treatment led to a greater response to treatment with both hormones than would be expected from their separate effects (Table 5). In another experiment in which thyroxine and cortisol were given intravenously no significant change occurred in the liver glucose 6-phosphatase activity during the succeeding 4 hr.

Thyroxine plus cortisol plus growth hormone. When growth hormone was given simultaneously with

Table 3. *Effect of treatment with growth hormone and cortisol on liver glucose 6-phosphatase activity of hypophysectomized rats*

(*a*) Experiments with animals eating *ad lib.*; (*b*) experiments with animals pair-fed.

Treatment of animal	Weight change (g./2 weeks)		Liver glucose 6-phosphatase activity (mean ± S.E.M.) (mg. of P/g. of liver)		(mg. of P/100 g. body wt.)	
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
None	6	−7	2·5±0·1	4·2±0·3	8·8±0·3	14·0±1·0
Cortisol	−26	−21	3·9±0·5	3·4±0·4	15·1±2·5	13·7±1·8
Growth hormone	39	+14	2·7±0·1	6·3±0·6	9·5±0·7	16·7±1·8
Cortisol and growth hormone	25	−6	3·6±0·2	6·6±0·5	15·5±0·8	17·7±1·7

Table 4. *Effect of daily treatment for 4 days with various hormones on the liver glucose 6-phosphatase activity of hypophysectomized rats fed* ad lib.

Treatment	Liver glucose 6-phosphatase activity (mean ± S.E.M.) (mg. of P/g. of liver)	(mg. of P/100 g. body wt.)
None	2·1±0·1	7·2±0·7
Cortisol	3·2±0·2	11·9±1·0
Thyroxine	4·6±0·3	14·5±0·9
Cortisol and thyroxine	8·8±0·6	24·8±1·4
Cortisol and thyroxine and growth hormone	8·6±0·6	27·7±1·5

Table 5. *Effect of administration of cortisol and of thyroxine on the liver glucose 6-phosphatase activity of hypophysectomized rats*

Period of treatment ...	Liver glucose 6-phosphatase activity (mean ± S.E.M.) 24 hr.		4 days	
Treatment of animal	(mg. of P/g. of liver)	(mg. of P/100 g. body wt.)	(mg. of P/g. of liver)	(mg. of P/100 g. body wt.)
None	2·1±0·1	8·1±1·0	—	—
Cortisol	2·6±0·1	9·6±0·2	—	—
Thyroxine	3·7±0·5	12·0±1·5	4·1±0·6	14·0±0·8
Cortisol and thyroxine	3·6±0·4	12·5±1·4	7·9±0·5	25·3±2·0

Table 6. *Liver glucose 6-phosphatase activity of rats treated in various ways*

Treatment of animal	Liver glucose 6-phosphatase activity (mean ± S.E.M.) (mg. of P/g. of liver)	(mg. of P/100 g. body wt.)
1. None	3·8±0·2	13·9±1·4
2. Hypophysectomized	2·6±0·2	8·8±0·6
3. Alloxan-diabetic	8·4±0·6	32·3±2·7
4. Hypophysectomized, alloxan-diabetic	2·7±0·4	7·8±0·8
5. Hypophysectomized, alloxan-diabetic, with growth hormone and cortisol	5·7±0·2	22·6±2·5

cortisol and thyroxine no greater effect on liver glucose 6-phosphatase activity was seen than with thyroxine and cortisol without growth hormone (Table 4).

Influence of alloxan diabetes on glucose 6-phosphatase in relation to the pituitary gland

The induction of alloxan diabetes in the normal rat causes a substantial rise in the liver glucose 6-phosphatase activity (Ashmore *et al.* 1954; Langdon & Weakley, 1955). The results in Table 6 show that such an effect of alloxan diabetes is not seen in hypophysectomized rats. When the alloxan-diabetic hypophysectomized rat was treated with both growth hormone and cortisol a highly significant ($P<0·01$) increase in liver glucose 6-phosphatase activity was found (Table 6).

Miscellaneous effects of hormone treatment of the hypophysectomized rat

In the experiments involving either a single treatment with hormones or daily treatment for 4 days, estimations of liver glycogen content and of blood sugar were carried out. No significant alterations from control values were found 24 hr. after a single treatment with cortisol and thyroxine either separately or together. After 4 days of treatment with thyroxine alone the blood sugar had fallen from 98 to 65 mg./100 ml., and the liver glycogen content had fallen from 1·5 to 0·3%. When both thyroxine and cortisol were given together, the blood-sugar value was the same as that with thyroxine alone (65 mg./100 ml.) and liver glycogen rose to a value of 2·2%.

DISCUSSION

The diminution in liver glucose 6-phosphatase activity we have observed to follow hypophysectomy in the rat was reported in a preliminary communication by one of us (Harper, 1956). This finding is not in agreement with the observations of Ashmore *et al.* (1956), who used large rats a few days after they had been hypophysectomized (conditions that would probably result in only small differences, cf. Fig. 2), but is in agreement with those of Reid, O'Neal & Lewin (1956).

The extent of the fall in liver glucose 6-phosphatase after hypophysectomy in the rat appears not to depend on the initial enzyme activity in the liver. In intact rats of 200 g. or more the enzyme activity falls as the animal grows older (Fig. 1), so that in such heavier animals the effect of hypophysectomy is less striking than it is in animals of less than 200 g. body wt. It is possible that the fall in enzyme activity with time, seen with intact rats of about 200 g. and upwards, is associated with a decline in pituitary activity, but no direct evidence for this view has been obtained.

The fact that starvation or food restriction causes a rise in the liver-enzyme activity in the normal rat, and a smaller one in the liver of the hypophysectomized rat (Table 2), complicates the interpretation of the results of experiments involving pair-feeding. A comparison of the results (Table 3) in feeding *ad lib.* and in pair-feeding suggests that the rise of liver-enzyme activity in response to growth hormone, or growth hormone plus cortisol, is associated with the restriction in food intake. The response per gram of liver was roughly proportional to the extent of the food restriction. Reid *et al.* (1956) also observed that there was a rise in the liver glucose 6-phosphatase activity of hypophysectomized rats treated with growth hormone and pair-fed with untreated hypophysectomized rats. Langdon & Weakley (1955) found no effect of treatment with growth hormone on the glucose 6-phosphatase activity of the liver in intact rats. It is possible that the rise in liver-enzyme activity brought about by starvation or food restriction is related to the increased rate of gluconeogenesis and may involve the secretory activity of either or both the adrenal cortex and the anterior pituitary gland.

Our most striking findings have been with thyroxine and with thyroxine plus cortisol (Table 5). Maley & Lardy (1955) observed that the administration of thyroxine to intact rats induced a rise in liver glucose 6-phosphatase activity, and Weber, Allard, de Lamirande & Cantero (1956) found that liver glucose 6-phosphatase activity is substantially increased in intact rats by the administration of cortisone. Ashmore *et al.* (1956)

found no diminution in concentration of the enzyme in the liver after adrenalectomy in the rat. Nevertheless, there was a fall in total activity in that organ which could be prevented by treatment with cortisol. In general our results for the effects of hypophysectomy in the rat, and the influence of hormone treatment of the hypophysectomized rat, are in agreement with what might be expected from the results with intact animals. The very high enzyme activity of liver tissue from hypophysectomized rats treated with both cortisol and thyroxine is not unexpected in view of the comparable response in the intact rat to treatment with these substances.

The fact that treatment with growth hormone, together with cortisol raises the low value for liver glucose 6-phosphatase activity seen in the hypophysectomized alloxan-diabetic rat (Table 4) does not justify the assumption that such an effect is independent of the availability of insulin in the animal, since the pancreas of the alloxan-diabetic rat has not necessarily lost all of its insulin-secretory tissue.

Ashmore *et al.* (1956) found no correlation between blood sugar and liver glucose 6-phosphatase activity, and have suggested that a high liver glucose 6-phosphatase activity might be associated with a low liver-glycogen content. However, our results with hypophysectomized rats indicate that the low liver-enzyme activity observed in this condition is associated with a low liver-glycogen content, whereas Weber *et al.* (1956) found that when rats are treated with cortisone high liver-glycogen values are observed to co-exist with high values for liver glucose 6-phosphatase activity. The effects of starvation, of treatment with thyroxine and of treatment with cortisol in raising liver glucose 6-phosphatase activity, and of hypophysectomy in inducing a fall, suggest the possibility that a rise in enzyme activity is associated with a rise in protein catabolism, and conversely. The effect of growth hormone in the pair-fed hypophysectomized rat would be compatible with such an idea since under such conditions protein may be catabolized in the liver for utilization in the muscles.

Our results suggest that the sluggishness of hepatic glycogenolysis observed by Cope & Marks (1934) may be associated with a depression in liver glucose 6-phosphatase activity. Our findings also suggest that thyroxine, cortisol and possibly growth hormone may all be involved in the influence of the anterior pituitary gland on such a process.

SUMMARY

1. Hypophysectomy of the rat is followed by a substantial fall in the liver glucose 6-phosphatase activity but no significant change in kidney glucose 6-phosphatase activity.

2. The liver glucose 6-phosphatase activity of the hypophysectomized rat rises as the result of starvation for 24 hr. but the rise is much less than that observed in a similarly treated intact rat.

3. The administration of ox-pituitary growth hormone to a hypophysectomized rat eating *ad lib.* does not change the total glucose 6-phosphatase activity in the liver, but if the treated hypophysectomized rat is pair-fed with an untreated one, the liver glucose 6-phosphatase activity rises.

4. The administration of either cortisol or thyroxine to the hypophysectomized rat is followed by a rise in liver glucose 6-phosphatase activity, the rise with thyroxine being detectable 24 hr. after one injection of the hormone. The administration of cortisol and thyroxine together induced a substantial increase in the liver glucose 6-phosphatase activity, bringing this value above that seen in the intact rat.

5. No relationship was observed to exist between liver glucose 6-phosphatase activity and liver-glycogen content or blood-sugar level.

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Hormonal Factors Affecting Glucose 6-Phosphatase Activity

2. SOME EFFECTS OF DIET AND OF ALLOXAN DIABETES IN THE RAT

By A. E. HARPER*
Department of Biochemistry, University of Cambridge

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A rise in liver glucose 6-phosphatase activity in rats which are made alloxan-diabetic or which are fasted has been reported (Ashmore, Hastings & Nesbett, 1954; Weber & Cantero, 1954; Langdon & Weakley, 1955). Because these and other investigations of the effects of hypophysectomy (Harper & Young, 1959) have shown that the food intake of the animals may greatly affect the glucose 6-phosphatase concentration in the liver, some effects of alterations in the diet, both in quantity and in composition, on liver glucose 6-phosphatase have been investigated. A preliminary account of some of these observations has already been published (Harper, 1956).

METHODS

Animals. White rats of the Wistar strain, bred in the Laboratory, were used. Unless otherwise stated, they consumed a stock diet *ad lib.* to such an extent that their body weight rose by 35–40 g. each week.

Alloxan diabetes was induced in rats by injecting intravenously 40 mg. of alloxan/kg. body wt. in animals weighing 150–250 g. Only those rats which after 1 week had a fasting blood sugar above 150 mg./100 ml. were used.

Estimation of glucose 6-phosphatase. Glucose 6-phosphatase activity was measured in a tissue homogenate as described in the preceding paper (Harper & Young, 1959). The values are expressed as enzyme concentration (mg. of P released/g. of tissue/10 min.) or as total activity/100 g. body wt.:

$$\frac{\text{Enzyme concentration} \times \text{liver wt. (g.)} \times 100}{\text{Body weight (g.)}}.$$

The problem of selecting the most suitable basis for expressing enzyme activity is discussed in the preceding paper (Harper & Young, 1959).

Miscellaneous. Blood sugar was estimated by the method of Somogyi (1952). Glycogen was determined by the method of Good, Kramer & Somogyi (1933). Moisture was determined by drying a weighed and homogenized portion of the liver to constant weight at 100°, and fat by ether extraction of the dried and ground liver. Nitrogen was determined on a portion of the homogenate by nesslerization after Kjeldahl digestion (Campbell & Hanna, 1937).

* Present address: Department of Biochemistry, University of Wisconsin, Madison 6, Wisconsin, U.S.A.

Table 1. *Effect of alloxan diabetes on the glucose 6-phosphatase activity in the liver and in the kidney of the rat*

	Liver glucose 6-phosphatase activity (mean ± S.E.M.)		Kidney glucose 6-phosphatase activity (mean ± S.E.M.)	
	(mg. of P/g.)	(mg. of P/100 g. body wt.)	(mg. of P/g.)	(mg. of P/100 g. body wt.)
Normal	4·0 ± 0·2	14·7 ± 0·4	3·8 ± 0·3	4·2 ± 0·2
Diabetic	7·9 ± 0·4	33·5 ± 2·2	6·3 ± 0·3	7·0 ± 0·2

Table 2. *Effect of starvation and of feeding glucose, fat or protein on glucose 6-phosphatase activity in the liver of the rat*

Treatment	No. of rats	Liver glucose 6-phosphatase activity (mean ± S.E.M.) (mg. of P/g. of liver)	(mg. of P/100 g. final body wt.)	(mg. of P/100 g. of original body wt.)
Fed *ad lib.*	9	4·7 ± 0·3	21·3 ± 1·9	—
Starved 24 hr.	9	8·0 ± 0·4	28·2 ± 1·2	25·2
Starved 48 hr.	8	8·4 ± 0·4	25·8 ± 1·8	22·5
Starved (24 hr.) and then fed with glucose for 24 hr.	12	5·3 ± 0·3	24·8 ± 1·4	22·1
Starved (24 hr.) and then fed with fat for 24 hr.	8	8·4 ± 0·3	31·0 ± 1·8	26·3
Starved (24 hr.) and then fed with protein for 24 hr.	8	8·0 ± 0·4	38·9 ± 1·9	33·8

RESULTS

Alloxan diabetes. A rise both in the concentration and in the total activity of glucose 6-phosphatase in the liver was found to follow the induction of alloxan diabetes (Table 1). In the kidney also a substantial increase in the enzyme concentration was observed as a result of the induction of alloxan diabetes and a smaller but nevertheless significant rise in the total activity/100 g. body wt. (Table 1, $P < 0{\cdot}01$).

Dietary changes. There was an increase in the glucose 6-phosphatase concentration in the liver of the starved rat with a smaller but still highly significant (P 0·01) rise in the total activity/100 g. final body wt. However, when the change in total activity was calculated on the basis of original body weight the difference was not significant ($P > 0{\cdot}1$) (Table 2). The values for animals starved for 48 hr. were not significantly different ($P > 0{\cdot}1$) from those for animals starved for only 24 hr.

When a rat which had been starved for 24 hr. was allowed to consume *ad lib.* glucose alone, butter alone or casein alone, the changes in glucose 6-phosphatase of the liver recorded in Table 2 were observed. After glucose alone there was a marked fall in glucose 6-phosphatase concentration but much less change in the total activity/100 g. body wt. The feeding of fat alone caused no change in enzyme concentration from the values for starved animals but the total activity/100 g. body wt. rose somewhat as opposed to the fall observed when starvation was continued for 48 hr. There was a significant difference (P 0·05) between the values for these two groups. When protein alone was fed, in the form of casein, again relatively little change was found in the enzyme concentration in the liver but the total enzyme activity/100 g. body wt. increased significantly ($P < 0{\cdot}01$) above that for the starved rat, the total activity being about the same as that found for the alloxan-diabetic rat.

The effects of these different types of treatment on the size of the liver and on the composition of this organ are shown for one experiment in Table 3. The most striking changes were observed in relative liver weight $\left(\frac{\text{liver wt.} \times 100}{\text{body wt.}}\right)$, glycogen content and nitrogen content. Starvation for 24 hr. caused a fall in relative liver weight, a depletion of glycogen and a rise in nitrogen concentration. Despite the higher nitrogen concentration there was actually some loss of nitrogen from the liver. The feeding of glucose to the starved rat resulted in a large accumulation of glycogen in the liver and a decrease in nitrogen concentration. The fall in total liver nitrogen was greater than that seen after starvation for 24 hr.

Table 3. *Composition of livers of rats on dietary experiment and relationship between liver nitrogen and glucose 6-phosphatase activity*

Values represent the means for a group of four rats, except that for fat, which was determined on pooled samples.

Treatment	Relative liver wt. (g./100 g. final body wt.)	Composition (%)				Liver nitrogen		Glucose 6-phosphatase (mg. of P/10 min./g. of liver N)
		Moisture	Fat	Glycogen (±S.E.M.)	Nitrogen (±S.E.M.)	(mg./100 g. final body wt.)	(mg./100 g. original body wt.)	
Fed *ad lib.*	4·5	71·8	3·6	4·2±0·9	3·35±0·05	151	—	122
Starved 24 hr.	3·7	72·6	4·1	0·03±0·01	3·08±0·02	147	132	133
Starved (24 hr.) and then fed with glucose for 24 hr.	5·0	71·0	2·2	7·9±0·7	2·52±0·1	127	112	220
Starved (24 hr.) and then fed with fat for 24 hr.	4·0	72·7	3·8	1·0±0·2	3·55±0·1	142	121	236
Starved (24 hr.) and then fed with protein for 24 hr.	4·9	71·5	2·8	1·6±0·2	3·70±0·1	182	163	235

Values for glucose 6-phosphatase activity/g. of liver nitrogen are also included in Table 3. In each case the activity reported on this basis was higher than that for the control group that was fed *ad lib.* Except for the group fed with casein, the rise was associated with a loss of liver nitrogen.

DISCUSSION

The observations on the effects of starvation and of alloxan diabetes on liver glucose 6-phosphatase are in agreement with those of Ashmore *et al.* (1954), Weber & Cantero (1954) and Langdon & Weakley (1955). In addition, the glucose 6-phosphatase activity of the kidney has been found to rise as a result of the induction of alloxan diabetes, and, as there is evidence that glucose is reabsorbed from the kidney tubules by an active mechanism which may well involve glucose 6-phosphatase, the increase in the enzyme activity in this organ may represent a physiological response of the kidney to conditions in which a rise in blood sugar imposes a greater metabolic load on the reabsorption mechanism.

The changes in glucose 6-phosphatase activity that occurred when the supply of each of the major dietary constituents was altered must be considered in relation to the effects of these treatments on relative liver weight and on liver composition.

Starvation for 24 hr. resulted in almost complete removal of glycogen from the liver and a fall in liver size. There was also some loss of nitrogen from the liver. This is most clearly seen when the nitrogen content of the liver is related to the original body weight. However, nitrogen was lost less rapidly than the other constituents with the result that the nitrogen concentration actually increased. Glucose 6-phosphatase was apparently lost even less rapidly than liver nitrogen generally because there was no fall in glucose 6-phosphatase activity/100 g. original body wt. (Table 2) and an increase in glucose 6-phosphatase activity/g. of liver nitrogen. The enzyme is evidently conserved during starvation, a situation similar to that observed for betaine-homocysteine transmethylase (Ericson & Harper, 1956). Weber & Cantero (1954) have suggested that the rise in glucose 6-phosphatase activity/g. of liver after starvation represents an adaptive change, but when the results are expressed in terms of total activity/100 g. original body wt. the increase is not significant and does not seem to warrant this conclusion.

The rapid fall in glucose 6-phosphatase concentration when the starved rat was fed with only glucose can also be explained as a result of changes in liver composition rather than in the total amount of enzyme present. Glycogen synthesis occurred rapidly when the starved animal was given glucose, relative liver weight increased, liver nitrogen was diluted and liver nitrogen/100 g. body wt. fell. The overall effect of these changes was that glucose 6-phosphatase concentration fell and glucose 6-phosphatase/g. of liver nitrogen rose (Tables 2 and 3), even though there was almost no change in total liver glucose 6-phosphatase activity/100 g. original body wt. (Table 2).

The effect of feeding fat did not greatly change the picture from that seen after starvation for 24 hr. There was, however, a significant difference between the value for the enzyme activity/100 g. body wt. after feeding with fat and that observed after starvation for 48 hr. There was also greater activity/g. of liver nitrogen than in the starved animal. This suggests that the protein-sparing action of fat (Munro, 1951) may make some of the amino acids from labile body proteins available for enzyme synthesis. It has since been found that if an adequate amount of protein is fed with fat in the absence of preformed glucose a highly significant rise in enzyme activity occurs (Freedland & Harper, 1957).

The effect of feeding protein alone to the starved rat was more clear-cut. Relative liver size increased, the amount of nitrogen in the liver increased, enzyme activity/g. of liver nitrogen was as high as that of the group fed only with fat, but, as total liver nitrogen was considerably higher, activity/100 g. body wt. (and/100 g. original body wt.) was highly significantly ($P < 0{\cdot}01$) greater than that of any of the other groups. Thus when the rat is deprived of glucose but is given an adequate amount of protein, a condition that would be expected to increase the rate of gluconeogenesis, there is a rise in its capacity to hydrolyse glucose 6-phosphate. This and observations of a similar nature in animals not previously starved (Freedland & Harper, 1957) suggest that the rise in enzyme activity represents an adaptive response to an inadequate supply of exogenous glucose. In this respect it is of interest that treatment with cortisone stimulates both gluconeogenesis and liver glucose 6-phosphatase activity (Harper & Young, 1959).

Also the metabolism of rats given protein alone resembles that of alloxan-diabetic rats, because in both instances much glucose arises in the liver through a process of gluconeogenesis. This increase in gluconeogenesis is accompanied, both in the alloxan-diabetic animal and in the protein-fed animal, by an increase in the total liver glucose 6-phosphatase activity/100 g. total body wt. The possibility therefore must be considered that the rise in liver glucose 6-phosphatase activity in the alloxan-diabetic rat does not result directly from a lack of insulin but is rather an indirect effect resulting from the depressed ability of the tissues

of such animals to utilize glucose, with a consequent rise of gluconeogenesis in the liver. Such a possibility is supported by the observation of Ashmore, Hastings, Nesbett & Renold (1956) that the administration of insulin can restore the blood-sugar level of alloxan-diabetic rats to normal before any change in glucose 6-phosphatase activity can be detected. The observations recorded in the present paper do not support the view that there need be any direct action of insulin on liver glucose 6-phosphatase.

SUMMARY

1. In the intact rat starvation for 24 hr. caused a rise in liver glucose 6-phosphatase concentration but relatively little change in the total glucose 6-phosphatase activity of the liver. This confirms the previous findings of others.

2. When the rat was allowed to eat glucose *ad lib.* after being starved for 24 hr. the liver glucose 6-phosphatase concentration fell almost to the value seen in the normal animal.

3. The changes in enzyme activity observed in the starved rat and in the rat fed with glucose after starvation for 24 hr. were apparent rather than real and can be satisfactorily explained as a result of changes in liver size and composition.

4. When the rat was allowed to eat fat or casein *ad lib.* after starvation for 24 hr. the glucose 6-phosphatase activity/100 g. of liver was the same as that of the starved animal. However, there was a small but significant rise in the total liver glucose 6-phosphatase activity/100 g. body wt. when the starved rat was fed with fat and a highly significant rise when it was fed with protein.

5. Alloxan diabetes caused a rise in liver glucose 6-phosphatase activity, as had been reported previously, and also a rise in kidney glucose 6-phosphatase activity.

6. The view is put forward that if sufficient protein is available, liver glucose 6-phosphatase activity in the rat rises in response to a deficiency of exogenous glucose.

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Metabolism of 11-Oxygenated Steroids

1. INFLUENCE OF THE A/B RING JUNCTION ON THE REDUCTION OF 11-OXO GROUPS

By I. E. BUSH and V. B. MAHESH
Department of the Regius Professor of Medicine, The Radcliffe Infirmary, Oxford

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In an earlier paper, it was suggested that the pattern of 11-oxo steroids and 11β-hydroxy steroids found in human urine might be explained if the well-known oxidation–reduction of steroid 11-oxygen functions *in vivo* was limited to those steroids having a more or less flat α-surface over the A and B rings (Bush, 1956). It was also suggested that this was a reasonable expectation, since reduction at C-11 is stereospecific and must presumably occur by association of the α-side of the steroid with the enzyme responsible for this reaction: steroids with a buckled or 'bent' A/B ring junction would not be likely to combine firmly with such an enzyme, which was already known to act rapidly on Δ^4-3-oxo steroids which have a roughly planar A/B ring system. It was suggested therefore that the substrate specificity of the 11β-dehydrogenase system in man was determined largely by the steric properties of 11-oxygenated steroids, particularly the A/B ring junction and

substituents on the α-side of the molecule. At the same time Hubener, Fukushima & Gallagher (1956) found that, with rat-liver homogenates, reduction of 11-oxo groups occurred rapidly with Δ^4-3-oxo steroids but not with several 3α-hydroxy-5β-(H) steroids (bent A/B junction); they suggested that the reaction was specific for Δ^4-3-oxo steroids although they had not tested the system with any 5α-(H) steroids (flat A/B junction).

This reaction seemed of interest because it appeared likely that adrenal 11-oxo steroids such as cortisone were not themselves biologically active on the tissues affected by the adrenocortical hormones, but were active when administered systemically only because of their rapid and complete reduction by the liver to the related 11β-alcohols (Bush, 1956; Peterson, Pierce, Wyngaarden, Bunim & Brodie, 1957). An alternative possibility in the light of the known reversible oxidation–reduction of steroid 11-oxygen functions was that such hormones exerted their action in a way similar to the coenzymes, in this case by acting as an essential cofactor in a redox system. This suggestion has been developed further by Talalay & Williams-Ashman (1958), who on the basis of experiments with oestradiol and placental *iso*citric dehydrogenase (Villee, 1955), suggest that since most steroid dehydrogenases in animal tissues can use both diphosphopyridine nucleotide and triphosphopyridine nucleotide [DPN^+ and TPN^+] as coenzyme, steroid hormones may in general exert their action by catalysing transhydrogenation from reduced diphosphopyridine nucleotide to triphosphopyridine nucleotide DPNH to TPN^+. They have shown that this catalysis of transhydrogenation is almost certainly the basis of the stimulation of placental *iso*citric dehydrogenase *in vitro* by oestradiol that was observed by Villee, and point out that the same dual requirement for pyridine nucleotide exists for the steroid 11β-dehydrogenase system of liver as for the oestrogen 17β-dehydrogenase. Despite the convincing explanation of Villee's results offered by Talalay & Williams-Ashman on this basis, our results (Bush & Mahesh, 1957, 1958*b*, *c*) and those of others (Glenn, Stafford, Lyster & Bowman, 1957; Huggins, Jensen & Cleveland, 1954) lead us to favour the alternative general theory, namely that the 11β-hydroxy steroid hormones, but not the related 11-ketones, are active *per se* at their site of action, and that the known oxidation–reduction of the 11-oxygen function of such steroids is not itself directly concerned in their mode of action.

Nevertheless, whichever theory is adopted the properties and specificity of systems responsible for oxidation–reduction of steroids at C-11 are of great interest, since they will probably play an important part, directly or indirectly, in determining the pharmacological activity of 11-oxygenated steroids. In the present paper we describe metabolic experiments in human subjects in which the main aim was to examine the part played by the steroid A/B ring junction in determining the substrate specificity of the steroid 11β-dehydrogenase *in vivo*. In addition, several other metabolic pathways of interest have been demonstrated.

METHODS AND MATERIALS

The steroids used in this study were administered to young healthy male subjects going about their normal business. In order to reduce the complications due to the presence of variable quantities of metabolites of endogenous steroids their adrenal secretion was suppressed to a low level by giving prednisone or prednisolone (1:2-dehydrocortisone or 1:2-dehydrocortisol) before and during the administration of the exogenous steroid precursors.

Samples (24 hr.) of urine were collected under toluene and preserved at 2–4°. Extraction was begun within 2–7 days of collection and all extracts and fractions from chromatograms were stored in the refrigerator between manipulations and kept out of 'fluorescent' lighting at all times.

Blood samples were collected in heparinized syringes and centrifuged immediately after collection. The plasma was drawn off and stored in the refrigerator after solid potassium oxalate had been added.

Chromatography was carried out at 25·5° by the procedure of Bush & Willoughby (1957). The solvent systems were: 1, benzene–methanol–water (2:1:1, by vol.); 2, toluene–methanol–water (4:3:1, by vol.); 3*a*, light petroleum–benzene–methanol–water (67:33:80:20, by vol.); 3*b*, light petroleum–toluene–methanol–water (67:33:85:15, by vol.); 4*a*, light petroleum–methanol–water (100:96:4, by vol.); 4*b*, light petroleum–methanol–water (20:17:3, by vol.). The light petroleum had b.p. 100–120°. Unless otherwise stated Whatman no. 2 filter paper was used. R_F values of reference compounds are given in Table 1.

Urine extracts were pre-fractionated by chromatography on strips of Whatman no. 3MM to give three fractions containing steroids of different polarity. Fraction 1 contained the least polar steroids, such as androsterone, fraction 2 steroids of medium polarity (e.g. 3α:11β-dihydroxy-5α-androstan-17-one) and fraction 3 the most polar steroids such as cortisol.

Reagents and materials

These were prepared and purified by the methods of Bush & Willoughby (1957) with the following modifications and additions.

Decolorite (The Permutit Co. Ltd., London, W. 4). This weakly basic resin was obtained as black irregular grains of mesh range −16 to +50. The resin was sieved with tap water to obtain the mesh range 24–50 and fine material removed by elutriation with tap water. The resin was then suspended in 3–4 vol. of N-NaOH and heated to 60–70° in an evaporating basin. It was stirred every 10–15 min. and the alkali decanted and sucked off after 3 hr. Trapped alkali was washed out by suspending in distilled water, stirring well, and decanting the water. This treatment with

Table 1. *Chromatographic properties of metabolites and their derivatives*

Compound	Configuration of 5-(H)	R_P or mobility in cm./hr.	Solvent system	Formula in Fig. 1
11β-Hydroxyandrosterone	α	0·16	3*b*	I
11β-Hydroxyaetiocholanolone	β	0·12	3*b*	I
Acetate of (I)	α	0·39	4*a*	II
Acetate of (I)	β	0·44	4*a*	II
11-Oxoandrosterone	α	0·33	3*b*	VI
11-Oxoaetiocholanolone	β	0·27	3*b*	VI
Acetate of (VI)	α	0·51	4*a*	III
Acetate of (VI)	β	0·55	4*a*	III
Androstane-3:11:17-trione	α/β	0·58	3*b*	V
*allo*Tetrahydrocortisol	α	12·5/20	1	VII
Tetrahydrocortisol	β	9·8/20	1	VII
Diacetate of (VII)	α	0·52	3*b*	VIII
Diacetate of (VII)	β	0·56	3*b*	VIII
*allo*Tetrahydrocortisone	α	16·4/20	1	IX
Tetrahydrocortisone	β	14·4/20	1	IX
Diacetate of (IX)	α	0·60	3*b*	X
Diacetate of (IX)	β	0·64	3*b*	X
Cortolone and β-cortolone	β	4·5/20	1	XI
3α:11β:17α:20ξ:21-Pentahydroxy-5α-pregnane	α	3·5/20	1	IV

NaOH was repeated twice. The resin was then treated similarly four times with N-HCl, washed with water on a Büchner funnel until the filtrate had a pH of 5–6, and suspended in 6–8 vol. of 85% aq. methanol (v/v) in which it was stirred mechanically for 16 hr. Finally the resin was stirred mechanically for 30 min. with excess of water and the water decanted: after a second treatment with water for 30 min. it was stored in a fresh lot of water in the cold room. During the final washing with 85% methanol and water much fine material is released and removed by stirring and decanting. The washed resin is stable for many months in the cold room but is rather friable. Stability is greatly impaired by traces of HCl remaining. This is best tested by examining the pH of the suspending fluid after a few days' storage; it should be 6 or above. Care was also taken not to crush resin particles with the glass stopper of its storage bottle: this produces fines which can be troublesome. It has recently been found that the hydroxide form is also suitable for extracting steroids and their conjugates: this form is conveniently prepared by reversing the order of the treatments with NaOH and HCl.

Sodium borohydride and lithium aluminium hydride. These were used as supplied.

21-*Acetoxy*-3α:17α-*dihydroxy*-5β-*pregnane*-11:20-*dione* (*tetrahydrocortisone* 21-*acetate*). This was supplied by Roussel Laboratories, m.p. 221–223°, and was chromatographically pure to at least 98%, moving 14·5 cm. in 20 hr. in system 1, after saponification.

3α:17α:21-*Trihydroxy*-5α-*pregnane*-11:20-*dione* (allo*tetrahydrocortisone*). Toluene-*p*-sulphonyl (tosyl) chloride (1 g.) was added to a solution of 21-acetoxy-3β:17α-dihydroxy-5α-pregnane-11:20-dione (1 g.) in dry pyridine (8 ml.) and left at room temp. overnight. Excess of reagent was decomposed with ice–water and the steroid extracted with ether–chloroform (1:1, v/v). The extract was dried with anhydrous Na_2SO_4 and evaporated at 45° (or below) under reduced pressure, giving a gum which, on trituration with ether, yielded crystalline 21-acetoxy-3β-tosyloxy-17α-hydroxy-5α-pregnane-11:20-dione (1·1 g.). The toluene-*p*-sulphonate (1·1 g.) was dissolved in acetic acid (30 ml.), heated under reflux for 2 hr. with anhydrous potassium acetate (2 g.) and kept for 24 hr. at room temp. The acetic acid was evaporated under reduced pressure at 45° (or below) and water (50 ml.) was added. The steroid was extracted with ether–chloroform (1:1, v/v) and the extract evaporated to a gum. This was dissolved in 0·067 N-NaOH in 80% aqueous methanol (100 ml.) and left in the dark under nitrogen for 21 hr. After acidification to pH 4·0 with acetic acid, the solution was evaporated under reduced pressure: the residue was dissolved in water (50 ml.) and extracted with ether–chloroform (1:1, v/v). The extract was evaporated and the residue dissolved in benzene for chromatography. A column of 100 g. of silica gel (2·5 cm. diam.; B.D.H. Ltd. 'for chromatography') was used and the steroid adsorbed on it from the benzene solution. The column was washed with benzene (500 ml.), benzene–ethyl acetate (2:1, v/v; 500 ml.), and ethyl acetate–methanol (1:1, v/v; 1500 ml.). The last solvent mixture was evaporated under reduced pressure and yielded 60 mg. of a solid, which was further purified by chromatography on six sheets (25 cm. × 45 cm. long) of Whatman no. 3MM paper in system 1 for 6 hr. The compound was located with blue-tetrazolium reagent on a 0·3–0·5 cm. wide strip cut out of each sheet and the appropriate zones of each sheet were extracted with ethyl acetate–methanol (2:1, v/v). Crystallization from ether yielded 51 mg. of material, m.p. 214–215° [*allo*tetrahydrocortisone 208–212° (Caspi, Levy & Hechter, 1953)], and chromatographically pure (the material moved 16·4 cm. in system 1 in 20 hr. compared with 16·4 ± 0·4 cm. for known *allo*tetrahydrocortisone on the same sheet).

3α:11β-*Dihydroxy*-5β-*androstan*-17-*one* (11β-*hydroxyaetiocholanolone*). A solution of 3α:17α-dihydroxy-5β-pregnane-11:20-dione (1 g.) in dry tetrahydrofuran (25 ml.) was added dropwise to a suspension of lithium aluminium hydride (0·5 g.) in boiling tetrahydrofuran (100 ml.), the addition taking about 10 min. Further tetrahydrofuran (50 ml.) was added and heating continued for 2 hr. Water (5 ml.) was added cautiously and the mixture brought to pH 4 with H_2SO_4. The solvent was evaporated to a small volume under reduced pressure and 100 ml. of water added. The mixture was extracted thrice with 300 ml. of

methylene chloride and the extract dried over Na_2SO_4 before evaporating under reduced pressure. The crude product was dissolved in 50% (v/v) aqueous acetic acid (300 ml.) and shaken with sodium bismuthate (20 g.) for 1·5 hr. in the dark. Aqueous sodium metabisulphite (100 ml., 10% w/v) was added and the mixture shaken for 30 min. After 3N-NaOH (500 ml.) had been added the steroid was extracted with ether–chloroform (1:1, v/v) and the extract washed with 3N-NaOH and water. On evaporation a white solid was obtained which crystallized from ethanol in prisms [m.p. 236–238°; 11β-hydroxyaetiocholanolone, m.p. 237–239°, Sarett (1948)]. The material was chromatographically pure and had an R_P in system 3*b* of 0·12 (11β-hydroxyaetiocholanolone 0·12 on the same sheet).

3α-Hydroxy-5β-androstane-11:17-dione (*11-oxoaetiocholanolone*). This was supplied by Roussel Laboratories, m.p. 188–189°. The R_P in system 3*b* was 0·27 (11-oxoaetiocholanolone from the M.R.C. Reference Collection had values of 0·27 on the same sheet).

3α:11β-Dihydroxy-5α-androstan-17-one (*11β-hydroxyandrosterone*). This was prepared by the method of Klyne & Ridley (1956) from 3β:17α-dihydroxy-5α-pregnane-11:20-dione. The m.p. was 198–200° and the R_P in system 3*b*, 0·16, in agreement with a reference standard (M.R.C. Steroid Collection).

3α-Hydroxy-5α-androstane-11:17-dione (*11-oxoandrosterone*). This was prepared by the method of Klyne & Ridley (1956) from the 21-acetate of the above starting material, and had m.p. 148–150°. The R_P in system 3*b* was 0·33, identical with a standard prepared from the above M.R.C. reference standard.

Chemical methods

Extraction of urinary steroids. Procedure 1 was identical with that described by Bush & Willoughby (1957).

Procedure 2 was that described briefly by Bush & Gale (1957). Urine (0·05 of a 24 hr. sample; 70–100 ml.) is percolated at 3 ml./min. through a column of Decolorite (1 cm. × 22 cm.) containing 15 ml. of resin (when measured under gravity packing in water) and the column is then washed with 40 ml. of water at the same rate. The free and conjugated steroids are then eluted with 80 ml. of methanol–aq. NH_3 solution (sp.gr. 0·88) (19:1, v/v). The eluate is evaporated under reduced pressure, with a wide-bore capillary (approx. 1 mm.) leak and a high-capacity condensing system with acid traps for about 5 min. with no external heating, and the evaporation then continued with the flask on a water bath at 40–45°. The residue is then dissolved in 20 ml. of 0·5M-sodium acetate–acetic acid buffer at pH 4·8, 0·5 ml. of succus entericus from *Helix pomatia* (about 40 000 Fishman units of glucuronidase) are added (Henry & Thevenet, 1952) and the mixture is incubated for 15–16 hr. at 45°. This solution was then extracted as in procedure 1.

Extraction of plasma. The heparinized and oxalated plasma was diluted with an equal volume of water, and 0·05 vol. of N-NaOH added (Short, 1957). The mixture was then extracted twice with 3 vol. of ether–ethyl acetate (2:1, v/v), the extracts were combined and washed once with 0·05 vol. of N-NaOH, and once with water. This extract was acidified with two drops of acetic acid, filtered through about 2 g. of anhydrous Na_2SO_4 and evaporated under reduced pressure at 35–40° with a very fine capillary leak. The flask was removed from the water bath and cooled under the tap as soon as the extract was reduced to 0·3 ml. or less, and the residue transferred with ethyl acetate and methanol to a conical centrifuge tube for evaporation with an air jet. This residue was run on chromatograms as described below.

Estimation of urinary α-ketols. This was carried out by the method of Bush & Willoughby (1957).

Estimation of urinary 17-oxo steroids. Fraction 2 from the pre-fractionation chromatogram was eluted and 0·8 of it deposited over the full width of a 2·5 cm. × 57 cm. strip of Whatman no. 2 paper in a zone about 6 cm. long at one end of the strip. The material was then concentrated by running up to the origin line with ethyl acetate–methanol (2:1, v/v) and the strip run, together with standards, in system 3*b* for 12–15 hr. after 5 hr. equilibration. This time of equilibration is longer than that used for sheets and has been found necessary to avoid 'edging' which makes quantitative estimation by the scanning method difficult. The strips were dried for 30–40 min. in the fume cupboard.

The dried strips were then dipped once over alkaline *m*-dinitrobenzene and heated as described by Bush & Willoughby (1957). The strips were left exposed to room air for 5 min. and then packed in groups of five, sandwiched between two strips of Whatman no. 2 paper (5·0 cm. × 56 cm.) which had been treated with alkaline *m*-dinitrobenzene and dried, shortly after each group of five chromatograms had been treated with the reagent. The exposure to air for 5 min. reduced the background colour; the sandwiching between the blank sheets of treated filter paper prevented the colour reaction with 17-oxo steroids from fading, thus allowing sufficient time for scanning the strips. The scanner and the filter used were the same as those used for the blue-tetrazolium method above.

Fraction 1 from the pre-fractionation chromatogram was treated exactly as fraction 2 except that the strips were run in system 4*a*, for 14 hr. after 5 hr. equilibration.

Estimation of Δ^4-3-ketones. This was carried out by the modified NaOH fluorescence reaction with aq. alkali (Bush, 1953*a*) but omitting triphenyl tetrazolium from the reagent. One-fifth of fraction 3 from the pre-fractionation chromatogram was run in system 2 for 6–7 hr. to obtain a slightly overrun chromatogram, in parallel with known standards in quantities ranging from 0·2 to 2·0 μg. Only cortisol was estimated since other Δ^4-3-oxo steroids usually found in urine were not detectable owing to suppression of adrenocortical secretion with prednisone. This method is based on visual matching with standards and has an error of ±15% in the optimum range.

Chromatographic identification of metabolites. The methods were similar to those described by Bush & Willoughby (1957). The following additional methods and modifications were used.

Oxidation with sodium bismuthate. On occasion it was found that considerable oxidation of 11β-hydroxyl groups to 11-oxo groups had occurred with this reagent. This varied considerably with different samples of bismuthate but could be reduced to a certain extent by carrying out the oxidation for 1 hr. with 25 mg. of sodium bismuthate in 1 ml. of 50% acetic acid (v/v). When the preservation of 11β-hydroxyl groups was essential to the identification, samples of bismuthate were used which had been tested

previously to show that they did not oxidize 11β-hydroxyl groups.

Selective reduction of 11-*oxo groups.* Reactive ketone groups were protected by making the ethylene ketals, after which the 11-oxo group was reduced with sodium borohydride (Appleby & Norymberski, 1955) and the 11-hydroxy compound (almost entirely 11β-) regenerated by hydrolysing the ketal with acid. The dry residue containing 20–50 μg. of steroid was refluxed with benzene (10 ml.), ethylene glycol (0·2 ml.) and toluene-*p*-sulphonic acid (5 mg.) for 3·5 hr. and the mixture evaporated under reduced pressure at 40–45°. Aq. NaOH (0·2N, 10 ml.) was added and the ketal extracted by shaking twice with ethyl acetate (30 ml.). The extract was washed twice with 5 ml. of water, dried by filtering through 1 g. of anhydrous Na_2SO_4, and then evaporated under reduced pressure, to dryness. The dry product was refluxed with tetrahydrofuran (10 ml.), sodium borohydride (15 mg.), and NaOH (2·5%, w/v; 1·0 ml.) for 10 hr. Water (10 ml.) was then added and the tetrahydrofuran evaporated under reduced pressure at 40–45°. The aqueous residue was extracted with ethyl acetate as above, washed, dried and evaporated. The dry reduction product was dissolved in acetic acid (2·0 ml.) and heated on a boiling-water bath for 30 min. Water (10 ml.) was then added and the mixture extracted twice with ethyl acetate (30 ml.). The extract was washed with saturated $NaHCO_3$ solution until evolution of gas had ceased, washed twice with water (5 ml.) and dried with anhydrous Na_2SO_4. The extract was evaporated as above (Antonucci, Bernstein, Lenhard, Sax & Williams, 1952; Allen, Bernstein & Littell, 1954).

This reaction gives an additional and highly specific indication of the presence of an 11-oxo group in a steroid, when used in conjunction with chromatographic techniques. Ketal formation can also be used for the demonstration of reactive ketone groups in the same way that acetylation reveals esterifiable hydroxyl groups. In this respect it is superior to thiosemicarbazide (Bush, 1953*b*), since the ketals are less polar (Reineke, 1956) and more soluble than comparable steroid thiosemicarbazones and thus usually more suitable for chromatography.

EXPERIMENTAL AND RESULTS

The procedure for experiments *A–E* was to give prednisone (5 mg. at 8.00 a.m., 12.30 p.m. and 8.00 p.m.) by mouth to a male subject for 3 days. On the second and third days 24 hr. urine samples were collected between 10.00 a.m. and 10.00 a.m. The second day's urine, sample 1, served as control. The third day's urine was designated sample 2. On the third day the compound under investigation was given at 10.00 a.m. In experiments *F* and *G* prednisolone (5 mg. at 8.00 a.m. and 8.00 p.m.) was given instead of prednisone and urine was collected on the third (sample 1) and fourth (sample 2) days. The dose was given on the fourth day at 10.15 a.m. in experiment *F* and at 9.00 a.m. in experiment *G*. Doses and results are given in Table 2 and the derivatives prepared for identification and their properties are summarized in Fig. 1 and Table 1.

Metabolism of 11β-*hydroxyaetiocholanolone* (*Experiment A*)

Routine estimation of urinary steroids was carried out by extraction procedure 1. One-twentieth of the urine sample of the third day was also extracted with ether–ethyl acetate without previous extraction and hydrolysis of the conjugates, and the steroid content of the extract estimated as above. This extract contained 10 μg. of 11-hydroxyaetiocholanolone (= 0·2 mg./24 hr.).

Identification of 11β-*hydroxyaetiocholanolone.* Fraction 2 from the pre-fractionation chromatogram of the extract of the urine of the third day was eluted and an amount equivalent to 0·01 of the 24 hr. sample was run on Whatman no. 2 paper in system 3*a* for 16 hr. spread over a width of 10 cm., with 11β-hydroxyaetiocholanolone in small amounts alongside the urinary material. The major component of the urinary fraction (detected on a thin strip cut from the sheet) and 11β-hydroxyaetiocholanolone both moved 11·5 cm. and gave violet colours with alkaline *m*-dinitrobenzene. The remaining part of the sheet was cut into small pieces and the zone containing the main component extracted with ethyl acetate–methanol (2:1, v/v) and the extract evaporated at 30–40° with a jet of air.

About 20 μg. of the material was run in system 3*b* for 3·5 hr. on the same sheet as a sample of 11β-hydroxyaetiocholanolone. Both substances had an R_F of 0·12, after detection with alkaline *m*-dinitrobenzene.

About 20 μg. of the urinary material and an equal amount of 11β-hydroxyaetiocholanolone were each oxidized with chromic acid and the products run in system 3*b* for 3·5 hr. A substance with an R_F of 0·58 was detected with alkaline *m*-dinitrobenzene, giving a blue–violet colour developing partly in the cold, identical in colour-reaction and position with the product from the reference substance (5β-androstane-3:11:17-trione).

About 60 μg. of the urinary material and a similar amount of 11β-hydroxyaetiocholanolone were each acetylated and the two products run in system 4*a* for 3·5 hr. A thin strip from the sheet was cut, taking a fraction of the lanes in which each product had been deposited and treated with alkaline *m*-dinitrobenzene. The acetate of the urinary material and of 11β-hydroxyaetiocholanolone both gave similar colours and had R_F values of 0·44. The remainders of the acetates of the urinary material and the reference steroid were extracted from the appropriate zones of the last chromatogram with ethyl acetate–methanol (2:1, v/v) and oxidized with chromic acid. The products were run in system 4*a* for 3·5 hr. and a part of the chromatogram was treated with alkaline *m*-dinitrobenzene.

Table 2. *Excretion of steroids after the administration of precursors*

In each experiment urine sample 1 is the control 24 hr. sample and sample 2 is the urine collected for 24 hr. after administration of the dose. Results are expressed in mg./24 hr., fully corrected. All subjects were males.

Expt.	*A*		*B*		*C*		*D*	*E*	*F*		*G*	
Age of subject (years)	29		24		21		29	24	33		24	
Precursor given	11β-Hydroxyaetiocholanolone		11-Oxoaetiocholanolone		11-Oxoaetiocholanolone		11β-Hydroxyandrosterone	11-Oxoandrosterone	Tetrahydrocortisone 21-acetate		*allo*Tetrahydrocortisone	
Dose (mg.)	100		45		45		50	50	60		50	
Urine sample	1	2	1	2	1	2	2	2	1	2	1	2
Urine vol. (ml.)	2400	1340	1060	1600	1500	1020	3100	1000	840	740	700	560
Steroid excreted												
Tetrahydrocortisol	1·9	0·81	0·43	0·77	0·53	0·39	0·47	0·64	1·3	2·1	0·71	0
Tetrahydrocortisone	1·5	0·90	0·72	1·3	0·80	1·1	0·37	0·53	0·86	17	0·73	0
*allo*Tetrahydrocortisol	0	0	0	0	0·23	0	0	0	0·78	1·0	0·64	13
*allo*Tetrahydrocortisone	0	0	0	0	0	0	0	0	0	0	0	1·5
Cortisol	0·29	0	0·11	0	0·057	0	0	0	0·021	0·022	0·014	0
11β-Hydroxyaetiocholanolone	0·11	47	0·16	0·93	0·27	0	0	0	0·24	0·26	0·21	0
11-Oxoaetiocholanolone	0·29	0·70	0·16	31	0·31	18	0	0	0·23	1·3	0·17	0
11β-Hydroxyandrosterone	0·31	0	0·23	0	0·43	0	31	6·3	0·20	0·25	0	1·2
11-Oxoandrosterone	0	0	0	0	0	0	0·87	21	0	0	0	0
Dehydro*epi*androsterone	0·90	1·3	0·13	0·15	0·08	0·35	0·62	0	0	0	0	0
Aetiocholanolone	1·0	0·74	0·41	0·87	0·71	1·1	2·0	0·53	3·0	2·7	2·4	1·9
Androsterone	0·33	0·40	0·36	0·83	0·93	0·61	2·5	0·79	3·5	2·5	2·1	2·1

Both the urinary material and the reference product gave pinkish colours developing partly in the cold, and had an R_F of 0·55, as with known 11-oxoaetiocholanolone 3-acetate. The remainders of the acetates on this chromatogram were extracted separately as above and each product was saponified. The products were then run in system 3*b* for 3·5 hr. and detected with alkaline *m*-dinitrobenzene. Both gave pinkish colours in the cold and had R_F values of 0·27, as did 11-oxoaetiocholanolone. This identification is summarized in Fig. 1 and Table 1.

Metabolism of 11-*oxoaetiocholanolone* (*Experiments B and C*)

11-Oxoaetiocholanolone was identified in fraction 2 of the urinary extract by the same general procedure used for the identification of 11β-hydroxyaetiocholanolone above.

Metabolism of 11β-*hydroxyandrosterone* (*Experiment D*)

The urine samples were extracted by procedure 2. 11β-Hydroxyandrosterone was identified by a procedure similar to those used above.

Metabolism of 11-*oxoandrosterone* (*Experiment E*)

11β-Hydroxyandrosterone was identified as above and 11-oxoandrosterone by preparing the derivatives shown in Fig. 1 and Table 1.

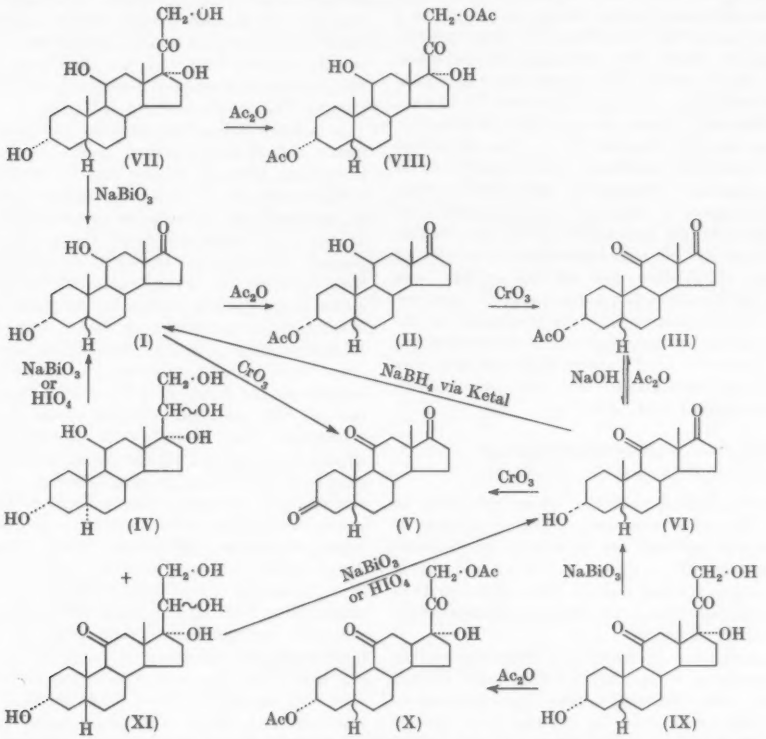

Fig. 1. Degradations used in the chromatographic identification of urinary steroids. (I), 3α:11β-Dihydroxy-5α/β-androstan-17-one; (IV) 3α:11β:17α:20α/β:21-pentahydroxy-5α-pregnane; (V) 5α/β-androstane-3:11:17-trione; (VI) 3α-hydroxy-5α/β-androstane-11:17-dione; (VII) 3α:11β:17α:21-tetrahydroxy-5α/β-pregnan-20-one; (IX) 3α:17α:21-trihydroxy-5α/β-pregnane-11:20-dione; (XI) 3α:17α:20α/β:21-tetrahydroxy-5β-pregnan-11-one. 5α/β means that both 5α-(H) and 5β-(H) isomers were degraded by the same routes. This is indicated in the figure by leaving the 5-H configuration ambiguous. Differentiation of these isomers is described in the text and summarized in Table 1. 20α/β means that such isomers were not separated but that probably both were present (Fukushima *et al.* 1955).

Metabolism of tetrahydrocortisone (Experiment F)

The urine was extracted by procedure 2. Plasma was separated from a blood sample drawn at 1.15 p.m. The concentrations of free steroids were extremely low and the results given below have a higher probable error. Tetrahydrocortisone was identified by the derivatives shown in Table 1 and by oxidation with sodium bismuthate to 11-oxoaetiocholanolone, which was identified as above. 3α:11β:17α:21-Tetrahydroxy-5β-pregnan-20-one (tetrahydrocortisol) was identified by the derivatives shown in Fig. 1 and Table 1 and by oxidation with sodium bismuthate to 11β-hydroxyaetiocholanolone, which was identified as described above.

11-Oxoaetiocholanolone was identified as above. 3α:17α:20:21-Tetrahydroxy-5β-pregnan-11-one (cortolone and β-cortolone, Fukushima *et al.* 1955) was detected when fraction 3 was run in system 1 for 20 hr. and a thin strip from the sheet cut out and treated with the periodate–Zimmermann reaction (Bush, 1955). The remainder was eluted and oxidized with sodium bismuthate to 11-oxoaetiocholanolone, which was identified as above.

Plasma steroids. Plasma (25 ml.) was extracted as above (Methods) and half of the extract run for 16 hr. in system 1. Treatment with alkaline blue-tetrazolium gave a blue spot in the position of tetrahydrocortisone estimated by eye to contain 0·9 μg. (uncorr.). No tetrahydrocortisol was found (<0·5 μg.). The other half of the extract was oxidized with sodium bismuthate and the product run for 6 hr. in system 3*b*. On treatment of the chromatogram with alkaline *m*-dinitrobenzene 0·8 μg. (uncorr.) of 11-oxoaetiocholanolone was found (visual estimation). No 11β-hydroxyaetiocholanolone was found (<0·3 μg.).

Metabolism of allo*tetrahydrocortisone (Experiment G)*

Blood was drawn from the antecubital vein at 12 noon. The urines were extracted by procedure 2. *allo*Tetrahydrocortisol was identified by preparation of the derivatives shown in Table 1 and Fig. 1, and by oxidation with sodium bismuthate to 11β-hydroxyandrosterone. 11β-Hydroxyandrosterone was identified as above.

On examination of fraction 3 from the second urine sample a compound running just behind cortolone was detected with the periodate–Zimmermann reaction. In system 1 it moved 3·5 cm. in 20 hr. at 25·5°. On oxidation with sodium bismuthate it yielded 11β-hydroxyandrosterone. These properties suggest very strongly that the original metabolite was the 5α-epimer of cortol or β-cortol (or both) (Fukushima *et al.* 1955), i.e. 5α-pregnane-3α:11β:17α:20α:21-pentol and its 20β-epimer.

Plasma steroids. These were estimated as in Expt. *F*. Amounts of 0·6 μg. (uncorr.) of *allo*tetrahydrocortisol and 0·6 μg. (uncorr.) of 11β-hydroxyandrosterone were found on the chromatograms. The related 11-ketones were not found (<0·4 μg. of *allo*tetrahydrocortisone and <0·2 μg. of 11-oxoandrosterone).

DISCUSSION

The results of these experiments confirmed that reduction of steroid 11-oxo groups in man is determined largely by the stereochemistry of the substrate, particularly the A/B ring junction, and not by any other obvious property of particular groups in ring A. Thus little if any reduction of their 11-oxo groups occurred with 11-oxoaetiocholanolone and tetrahydrocortisone, both of which have the buckled A/B ring junction typical of 5β-(H) steroids. On the other hand, the 11-oxo groups of their 5α-epimers (11-oxoandrosterone and *allo*tetrahydrocortisone) with flat A/B ring junctions were extensively reduced, especially with the latter steroid. This is summarized in Fig. 2.

It is more difficult to estimate the quantities of minor metabolites derived from the exogenous precursors because of the day-to-day and individual variation of the endogenous contribution to the metabolites. In order to minimize this source of error, we have calculated the probable endogenous contribution to each metabolite under consideration by multiplying the quantity excreted in the control sample by the ratio (excretion in sample 2)/(excretion in sample 1) with respect to an independent group of metabolites known from other work to have excretion rates which, in any individual, are closely correlated with the excretion rate of the metabolite under consideration. In considering the excretion of 11β-hydroxyaetiocholanolone in Expt. *B*, for instance, the figure for sample 1 is 0·16 mg./24 hr. (Table 2). Since this metabolite is normally closely correlated with the summed excretion rates of tetrahydrocortisone and tetrahydrocortisol (Dorfman, 1954; Roberts & Szego, 1955), we note that this sum is 1·15 mg./24 hr. in sample 1 and 2·07 mg./24 hr. in sample 2; multiplying 0·16 by the ratio 2·07/1·15 gives the probable endogenous contribution to the 11β-hydroxyaetiocholanolone in sample 2, namely 0·29 mg./24 hr. Since the observed figure in sample 2 was 0·93 mg./24 hr., it is probable that 0·64 mg. was derived from the exogenous precursor. Similarly, in considering the excretion of tetrahydrocortisol in Expt. *F*, *allo*tetrahydrocortisol was taken as the independent metabolite, giving a correction factor of 1·0/0·78 and the probable endogenous contribution to tetrahydrocortisol in sample 2 as $1{\cdot}3 \times 1{\cdot}0/0{\cdot}78 = 1{\cdot}7$ mg./24 hr. In Expts. *D* and *E* the control urines were not

analysed because of losses in the collection and values of 0·16 mg./24 hr. (mean of all other samples) for 11β-hydroxyandrosterone and of 0·0 mg./24 hr. for 11-oxoandrosterone were assumed.

This type of calculation is the basis for the figures given in Table 3, where the probable yield of metabolites from the exogenous precursors is expressed in each case as the percentage of the dose administered. While this procedure improves upon the more usual method of basing calculations on the uncorrected values of the control samples, it does not avoid the error due to day-to-day variation of the relative proportions of the endogenous metabolites. Thus the exogenous contribution to tetrahydrocortisol in sample 2 of Expt. *F* seems of questionable significance, while the apparently

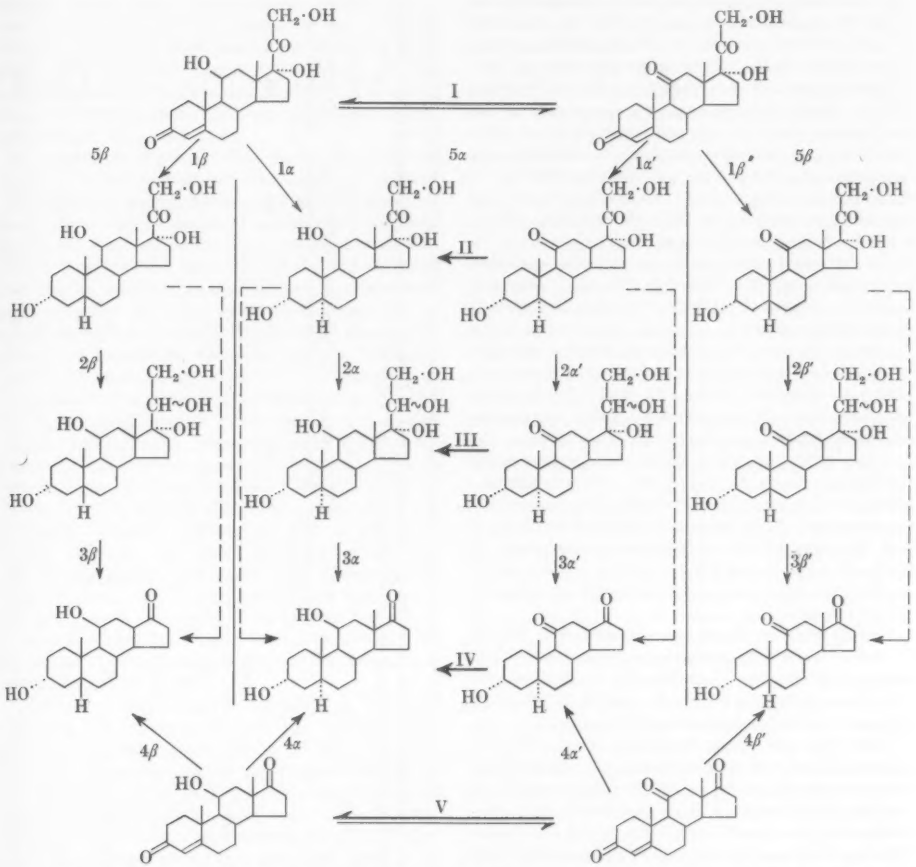

Fig. 2. Summary of the probable main pathways of metabolism of cortisol and 11β-hydroxyandrost-4-ene-3:17-dione condensed to single lines of 5α-(H) and 5β-(H) metabolites. It is known from other work that reaction 2 may precede 1; and it is possible that reaction 3 may precede 2, but not 1 to any great extent (Dorfman, 1954). Routes II and IV were demonstrated here for the first time; also routes 2β′, 2α and/or 2α′, and 3α and/or 3α′. Other routes have been demonstrated previously or are reasonable inferences from our results (Dorfman, 1954; Lieberman & Teich, 1953; Roberts & Szego, 1955). For simplicity reactions 2 and 3 have been drawn in sequence; the possibility that the 20-hydroxy steroids are not obligatory intermediates in the metabolic pathway from C_{21} to C_{19} steroids is indicated by the dashed arrows.

significant increase in excretion of 11β-hydroxyaetiocholanolone in Expt. *B* is rendered doubtful by the absence of this substance from sample 2 of Expt. *C*. On the other hand, the exogenous origin of most of the 11β-hydroxyandrosterone in sample 2 of Expt. *G*, and of 11-oxoaetiocholanolone in sample 2 of Expt. *F*, seems beyond reasonable doubt.

While the assumptions underlying the above procedure are well supported by other work on steroid metabolism, a stricter test of the significance of the supposed minor metabolites of the exogenous precursors can be made by assuming complete independence of the excretion rates of the different metabolites and applying Student's *t* test (Fisher, 1946). These calculations confirm the above conclusions, even though the assumption of independence of excretion figures is certainly an exaggeration, and rules out the possibility of applying any correction to the values of the control samples (i.e. samples 1). Thus the excretion of 11-oxoaetiocholanolone in Expt. *F*, sample 2, is 5·6 times the mean excretion in the samples available for comparison (*A*1, *B*1, *C*1, *F*1, *G*1), giving a value for *t* of 15·8 ($P < 0.025$). The excretion of 11β-hydroxyandrosterone in Expt. *G*, sample 2, is 7·5 times the mean excretion of the nine samples available for comparison (i.e. excluding *D* and *E*), giving a value for *t* of 6·4 ($P < 0.05$). In this case the difference between samples 1 and 2 can also be compared with a reasonable number of controls; in Expt. *G* it is +1·2 mg./24 hr., as against a mean for Expts. *A*, *B*, *C* and *F* of −0·23 mg./24 hr., giving a value for *t* of 7·0 ($P < 0.05$). The *t* values for the excretion of 11-oxoaetiocholanolone in Expt. *A* and for that of 11β-hydroxyaetiocholanolone in Expt. *B* are 6·4 and 6·9 ($P < 0.05$). Other minor metabolites gave *t* values of negligible significance ($P = 0.1$–0.4).

The 20-hydroxy metabolites described in Expts. *F* and *G* were not estimated quantitatively. The amount of cortolone found in the control sample was about one-tenth of that in sample 2; *allo*cortol was not detectable in the control sample.

Only with *allo*tetrahydrocortisone was complete suppression of endogenous adrenocortical secretion observed, as judged by complete disappearance of metabolites of cortisol from the urine. This contrasts with the experiments of Savard & Goldfaden (1954), who reported that tetrahydrocortisol had suppressed the endogenous adrenocortical secretion of two post-menopausal women. However, it is not clear from their paper how sensitive were their methods for estimating cortisol, nor were there details of control specimens of urine from their subjects. In view of the known biological activity of *allo*tetrahydrocortisol (Hechter, 1955; Silber, personal communication) and the inactivity of

Table 3. *Percentage recovery of identified metabolites and unchanged precursors from urine*

Recoveries are given as percentage of dose based on the fully corrected figures of Table 1. In Expt. *F* the dose and yield of metabolites are expressed in terms of the free steroid; the 21-acetate was administered. The urines were samples 2 (see Table 1).

Experiment	*A*	*B*	*C*	*D*	*E*	*F*	*G*
Precursor given	11β-Hydroxyaetiocholanolone	11-Oxoaetiocholanolone	11-Oxoaetiocholanolone	11β-Hydroxyandrosterone	11-Oxoandrosterone	Tetrahydrocortisone	*allo*Tetrahydrocortisone
Dose (mg.)	100	45	50	50	50	54	50
Steroid excreted	Group at C-11						
Tetrahydrocortisol	·OH	—	—	—	—	⩽0·72	—
Tetrahydrocortisone	:O	—	—	—	—	28	—
*allo*Tetrahydrocortisol	·OH	—	—	—	—	—	26
*allo*Tetrahydrocortisone	:O	—	—	—	—	—	3·0
11β-Hydroxyaetiocholanolone	·OH	⩽1·6	0	—	—	0	—
11-Oxoaetiocholanolone	:O	69	36	—	—	1·9	—
11β-Hydroxyandrosterone	·OH	—	—	62	12·0	0	2·4
11-Oxoandrosterone	:O	—	—	1·7	42	—	—
Total recovery of identified metabolites	⩾48	⩾71	36	64	54	⩾33	31

tetrahydrocortisol, our results are intelligible in view of the observed reduction of *allo*tetrahydrocortisone to *allo*tetrahydrocortisol and its appearance in the blood stream, although its concentration in plasma was low (about 7 μg./100 ml.; corr.).

Estimation of plasma steroids was rather unsatisfactory because of the low concentrations present. However, the compounds found suggested that the plasma ratios of 11-oxo/11β-hydroxy forms were similar to the ratios observed with the urinary metabolites. Identification was based on the chromatographic properties of the α-ketols themselves and of their oxidation products with sodium bismuthate. Since the normal plasma concentrations of these α-ketols are too low to be detectable by the above methods the identifications were considered satisfactory.

It might be objected that these observations admit of other interpretations, since the urinary metabolites of a steroid precursor are the result of many competing processes of transfer and enzymic action; or that reduction had occurred with the 5β-(H) steroids but that the 5β-(H)-11β-alcohols had been metabolized to undiscovered metabolites, or conjugated in some way not amenable to hydrolysis by our methods. There are no reasonable grounds at present for accepting such an explanation of our findings. Furthermore, the recovery of recognized metabolites (or unchanged precursor) was high (Table 3) and in Expts. *A* and *D* the related 11β-hydroxy steroids were themselves administered and recovered in comparable yield from urine by the same methods. Another possible objection might be that the redox potentials of the 11-oxygen function differed in the 5α-(H) and 5β-(H) steroids, so that although oxidation–reduction occurred the 11-ketones of the latter predominated in the steady-state. This interpretation is not only extremely unlikely, but disproved in the case of the C_{19} precursors in which administration of the 11β-alcohols yielded only the unchanged precursors in the urine.

While it is reasonable to expect that enzymic reduction of 11-oxo groups to 11β-hydroxyl groups by an enzyme specific for Δ^4-3-oxo steroids would be impossible, or at most extremely slow, with 5β-(H) steroids (A/B junction bent) it is not certain that a single enzyme is responsible for this reaction. There is still no evidence to exclude the possible existence of several enzymes capable of reducing the 11-oxo groups of 3α-OH-5α-(H) steroids and others, but until proved otherwise it seems reasonable to adopt a working hypothesis that the hepatic system is in fact based upon one steroid 11β-dehydrogenase, or at most a set of 11β-dehydrogenases with very similar properties of their active regions in those parts which are opposed to the A and B rings in the enzyme–substrate complex. Thus there is direct or indirect evidence showing that the reaction occurs *in vivo* or *in vitro* with steroids containing almost every known natural side chain at C-17 as well as with 17-oxo steroids (Burton, Keutman & Waterhouse, 1953; Burstein, Savard and Dorfman, 1953*a*, *b*; Savard, Burstein, Rosenkrantz & Dorfman, 1953; Hubener *et al.* 1956). On the other hand, there is similar evidence showing that with a variety of side chains at C-17 and with 17-oxo steroids the rules for substrate specificity are the same as far as deformations or substitutions in the AB ring system are concerned.

It may also be objected that rules of substrate specificity cannot be deduced from experiments *in vivo* since differences in distribution and transport of these steroids may be the basis of the differences observed in their metabolism. Two arguments seem to remove this objection from serious consideration: First, our results with 11-oxoaetiocholanolone and tetrahydrocortisone, and previous work with cortisone itself and 2α-methylcortisone, parallel very closely the observations of others with rat-liver homogenates, and with microsome fractions from rat liver, where differences in distribution and availability of the substrates are minimized (Glenn *et al.* 1957; Hubener *et al.* 1956). Secondly, very large differences in the solubility and other properties of such steroid substrates which would be expected to influence their transport and distribution have made no difference in the striking effects observed with differences in the AB ring system (e.g. C_{19} and C_{21} steroids, except for the rather inefficient reduction of 11-oxoandrosterone); on the other hand steroids with similar properties, and known to be metabolized, conjugated, and excreted at similar rates and by similar pathways, still show gross differences in reduction of 11-oxo groups according to the steric properties of their AB ring systems (e.g. cortisone, tetrahydrocortisone and *allo*tetrahydrocortisone).

The findings reported here are a reasonable explanation of the pattern of 11β-hydroxy and 11-oxo steroid metabolites found in normal human urine which was remarked upon previously (Bush, 1956). The suggested pathways of metabolism are generalized in Fig. 3. However, A. A. Sandberg & R. Slaunwhite (personal communication) have suggested that 11β-hydroxy-Δ^4-3-oxo steroids are preferentially reduced to 5α-(H)-3α-ols and 11-oxo-Δ^4-3-oxo steroids to 5β-(H)-3-ols. It will be shown in subsequent papers that this effect is definitely observable but it appears at present to be too small to account for the normal urinary pattern.

Thus it is clear from our results that the 5β-(H)-3α:11β-dihydroxy steroids are neither reduced nor oxidized appreciably at C-11. It follows that 5β-(H)-3α:11β-dihydroxy steroids must be derived

from the reduction of an 11β-hydroxy-Δ^4-oxo steroid precursor. Even if all the *allo*tetrahydrocortisol (5α-H) in human urine were derived from direct reduction of cortisol, and none via reduction of *allo*tetrahydrocortisone, it would appear from the usual predominance of tetrahydrocortisol (5β-H) that reduction of C_{21}-11β-hydroxy-Δ^4-3-oxo steroids to the 5β-(H)-3α-ols was favoured over their reduction to 5α-(H)-3α-ols (Dorfman, 1954; Bush & Willoughby, 1957).

Further work on oxidation–reduction of steroid 11-oxygen functions is in progress, some of which has been reported briefly (Bush & Mahesh, 1958*a–c*). To avoid possible confusion it is worth noting here that, as suggested by Morris & Williams (1953) among others, there is some evidence that a second system exists, more widely distributed in the peripheral tissues, in which the steady state is normally such as to produce a net oxidation of 11β-hydroxy steroids to the related 11-ketones. The substrate specificity of this second system is probably rather different from the hepatic system. Thus 9α-fluorocortisone was reduced completely to 9α-fluorocortisol after oral administration, but although it was metabolized by pathways otherwise identical with those of cortisone, the corresponding 9α-fluoro-11-ketones were not found (Bush & Mahesh, 1958*b*). More significantly the oxidation of endogenous cortisol (i.e. the small amount secreted before complete suppression of adrenocortical secretion had occurred) was inhibited, so that its normally predominant 11-oxo metabolites were only excreted in traces, while the normally less plentiful 11β-hydroxy metabolites were excreted in amounts which were low but far greater than those of the related 11-ketones. In this instance it would be unreasonable to suppose that the oxidative, but not the reductive, reaction

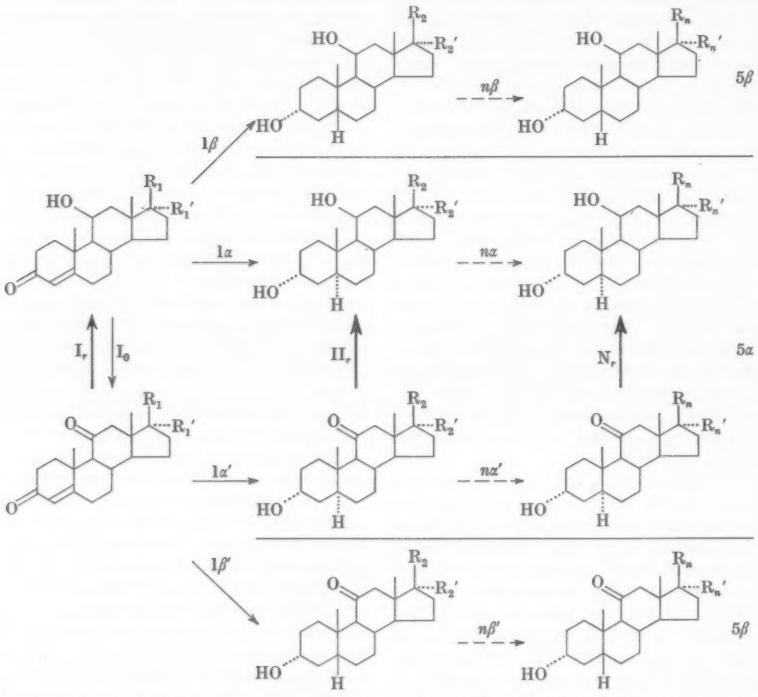

Fig. 3. Hypothetical generalization of the routes of metabolism shown in Fig. 2. Present work suggests that reactions I_r, II_r and N_r will not occur when large α-substituents at C-1, C-2, C-9 and C-12 are present; substituents at other positions, or 1-, 2- and 12-β substituents, probably do not stop these reactions. These suggestions are based partly on ordinary stereochemical reasoning and partly on results obtained with 2α-methyl steroids and 1:2-dehydro steroids (Bush & Mahesh, 1957, 1958*a*, *c*). The representation of substituent groups by R_1, R_2, etc. does not imply that these are necessarily altered in the course of the reactions.

due to the hepatic enzyme was inhibited by 9α-fluorocortisol, and one is left with the strong supposition that the system normally responsible for the well-known net oxidation of steroid 11β-hydroxyl groups has properties differing from those of the reducing system of the liver. This supposition is now under further examination.

SUMMARY

1. An earlier hypothesis that reduction of steroid 11-oxo to 11β-hydroxy groups *in vivo* was largely dependent on the stereochemistry of the A and B rings has been tested by studying the metabolism of various 3α-hydroxy steroids.

2. 11-Oxoandrosterone, 11β-hydroxyandrosterone, 11-oxoaetiocholanolone, 11β-hydroxyaetiocholanolone, tetrahydrocortisone and *allo*tetrahydrocortisone were given by mouth in doses of 40–100 mg. to young male volunteers, together with prednisone or prednisolone to suppress endogenous adrenocortical secretion. Steroids in blood plasma and urine were estimated and identified by chromatographic methods.

3. The proportions of the major metabolites of 11-oxoandrosterone (5α-H) and of *allo*tetrahydrocortisone (5α-H) the 11β-hydroxy (reduced) form were 23 and 91% respectively. No 11β-hydroxy metabolites of the other steroid precursors (5β-H) were detected (<2% of their major metabolites).

4. The C_{21} 3α-hydroxy steroids gave rise to small but significant amounts of the related 17-oxo steroids and 20ξ-hydroxy steroids.

5. It is suggested that the normal pattern of 11-oxo and 11β-hydroxy steroids in human urine, in which little or no 11-oxo-5α-(H) steroids can be found while the 11-oxo-5β-(H) metabolites are more plentiful than 11β-hydroxy-5β-(H) metabolites, is largely accounted for by the ease with which the 11-oxo-5α-(H) metabolites are reduced to the related 11β-alcohols.

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Metabolism of 11-Oxygenated Steroids

2. 2-METHYL STEROIDS

By I. E. BUSH and V. B. MAHESH
Department of the Regius Professor of Medicine, The Radcliffe Infirmary, Oxford

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In previous papers we have explained our reasons for studying the metabolism of 11-oxygenated steroids, and especially the interconversion of steroid 11β-alcohols and 11-ketones (Bush, 1956; Bush & Mahesh, 1958*a*, 1959*a*). The 2α-methyl steroids are particularly interesting because of a peculiar feature of their biological activity. The natural adrenocortical steroids with biological activity show a fairly constant relationship between the activity of 11-ketones such as cortisone (17α:21-dihydroxypregn-4-ene-3:11:20-trione) and the related 11β-alcohols, in this case cortisol (11β:17α:21-trihydroxypregn-4-ene-3:20-dione). In most biological assays, and particularly those related to so-called glucocorticoid activity in mammals, the 11-ketones have 0·3–0·5 time the activity of the 11β-alcohols. In these cases the 11-ketones and 11β-alcohols are readily interconvertible *in vivo* and in the steady state the latter have by far the greater concentration in blood (Bush, 1956; Peterson, Pierce, Wyngaarden, Bunim & Brodie, 1957). On oral administration of cortisone, and of other steroid 11-ketones having a relatively flat α-surface (Bush & Mahesh, 1957, 1959*a*), reduction is so efficient that little or no unchanged 11-ketone reaches the blood, and, with the exception of 5β-(H)-3α-hydroxy metabolites, all the urinary metabolites are largely in the 11β-hydroxy form.

With 2α-methylcortisol and 2α-methylcortisone, however, this relation broke down and the former was found to be from 10 to 50 times as active as the latter in assays for both glucocorticoid and mineralocorticoid activity (Liddle, Richard & Tomkins, 1956; Dulin, Bowman & Stafford, 1957). Peterson *et al.* (1957) suggested that this might be due to a failure of the liver to carry out the usual reduction of the 11-oxo group and it was shown that this was so both *in vivo* in man (Bush & Mahesh, 1957) and *in vitro* with rat-liver microsomes (Glenn, Stafford, Lyster & Bowman, 1957). This exceptional behaviour seemed to offer an excellent opportunity of deciding whether the pharmacological action of the 11-oxygen function in the 11-oxygenated adrenocortical steroids was confined to the 11β-hydroxyl group or depended upon the oxidation–reduction reaction undergone by this oxygen function (Bush, 1956; Bush & Mahesh, 1959*a*). In order to have sure grounds for adopting the former hypothesis, however, it was necessary to examine all the metabolites of 2α-methylcortisone and to compare its absorption with that of 2α-methylcortisol. To obtain further information on the factors determining oxidation–reduction at C-11, the metabolism of 2α-methyladrenosterone (2α-methylandrost-4-ene-3:11:17-trione) was also investigated. These experiments are described in this paper; experiments on cortisone and adrenosterone were carried out by the same methods to enable a direct comparison to be made between the methyl-substituted and unsubstituted precursors.

METHODS

Materials and reagents

These were prepared by the earlier methods (Bush & Mahesh, 1959*a*; Bush & Willoughby, 1957), except for the following.

2α-Methylcortisone (17α:21-*dihydroxy*-2α-*methylpregn*-4-*ene*-3:11:20-*trione*) *and* 2α-*methylcortisol* (11β:17α:21-*trihydroxy*-2α-*methylpregn*-4-*ene*-3:20-*dione*). These were gifts from the Upjohn Co., Kalamazoo, Mich., U.S.A. Purity was high judged by chromatography of the esters and the derivatives prepared from them (see below). Less than 2% of any single steroid impurity giving fluorescence with NaOH, reduction of alkaline blue tetrazolium, or a 17-oxo steroid from oxidation with sodium bismuthate was present.

2α-Methyladrenosterone (2α-*methylandrost*-4-*ene*-3:11:17-*trione*). 2α-Methylcortisone (60 mg.) was dissolved in 50 ml. of 50% (v/v) acetic acid and 1 g. of sodium bismuthate added. The flask was screened with black paper and shaken for 1·25 hr., after which 10 ml. of 10% sodium metabisulphate was added and the flask shaken a further 0·5 hr. The mixture was brought to pH 5·0 with 4N-NaOH, cooled in ice, and extracted thrice with 3 vol. of ether–chloroform (1:1, v/v). The combined extract was washed twice with 0·1 vol. of N-NaOH and then twice with 0·1 vol. of water. The extract was dried with about 5 g. of anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure at 40–45°. The residue was recrystallized from ethanol to give 41 mg. of a crystalline product which was chromatographically pure to 2% of any single impurity (m.p. 212–214°; uncorr.).

Cortisone. A dry, powdered preparation of the free alcohol was used (gift from Dr L. H. Sarett, Merck and Co., Rahway, N.J., U.S.A.). This was chromatographically pure to 1% (or less) of any single impurity as judged by the

Table 1. *Excretion of normal steroid metabolites in Experiments A–E*

Prednisone (15 mg.) or prednisolone (10 mg.) was given on both days of the urine collections. *C*, control day; T_1, first estimation; T_2, second estimation approximately 1 month after T_1. The results are expressed as mg./24 hr.

Experiment	*A*		*B*		*C*			*D*		*E*		
Precursor given	Cortisone		2α-Methyl-cortisone		2α-Methyl-cortisol			Adrenosterone		2α-Methyl-adrenosterone		
Dose (mg.)	100		50		50			50		40		
Urine sample	*C*	T_1	T_1	T_2	T_1	T_2		*C*	*T*	T_1	Recovery value (%)	Method of estimation
Steroids excreted												
Tetrahydrocortisol	0·79	13	1·7	1·4	1·4	1·1		1·4	0·86	0·86	70	Scanning; blue tetrazolium
Tetrahydrocortisone	1·0	21	2·4	2·3	2·0	1·9		1·0	0·87	0·53	75	
*allo*Tetrahydrocortisol	0·57	7·7	0·86	0·72	0·86	0·86		0·57	0·57	0·57	70	
20-Dihydrocortisol	<0·01	0·43	0·014	—	0·010	—		—	—	—	70	Visual; NaOH fluorescence
20-Dihydrocortisone	<0·01	0·05	<0·01	—	—	—		—	—	—	70	
Cortisol	<0·01	0·29	<0·01	—	—	—		0·014	0·01	0·029	70	
Cortisone	<0·02	0·021	<0·02	—	—	—		—	—	—	70	
11β-Hydroxyaetiocholanolone	0·21	1·6	0·63	0·55	0·44	0·33		0·20	1·6	<0·25	80	Scanning; alkaline *m*-dinitrobenzene
11-Oxoaetiocholanolone	0·36	3·3	0·94	0·75	0·48	0·34		0·26	4·1	<0·15	80	
11β-Hydroxyandrosterone	0·24	0·88	0·50	0·36	0·36	0·30		0·23	12	<0·25	80	
Dehydroepiandrosterone	<0·06	<0·06	2·0	1·4	1·3	1·0		<0·06	<0·06	<0·06	80	
Aetiocholanolone	2·4	1·7	3·4	2·8	2·9	2·0		2·3	3·1	1·8	85	
Androsterone	2·8	2·5	3·3	2·4	3·3	2·8		3·2	3·1	2·0	85	

alcohol, the 21-acetate, and oxidation with sodium bismuthate to adrenosterone.

Adrenosterone (*androst-4-ene-3:11:17-trione*). This was a gift from Dr L. H. Sarett and was chromatographically pure to 1% (or less) of any single 17-oxo steroid or impurities giving a fluorescence with NaOH.

11β-*Hydroxy-2α-methylandrostenedione* (11β-*hydroxy-2α-methylandrost-4-ene-3:17-dione*). This was prepared in amounts of 25–50 μg. as required for chromatographic correlations by oxidizing 2α-methylcortisol with sodium bismuthate [1·0 ml. of 50% (v/v) acetic acid with 25 mg. of $NaBiO_3$].

3β:11β-*Dihydroxy-2α-methyl-5α-androstan-17-one* (Chart 3). 2α-Methylcortisol (3 mg.) was hydrogenated in acetic acid (2 ml.) in the presence of activated platinum oxide (1 mg.) for 8 hr. and the product extracted with ethyl acetate. The extract was evaporated at 40–45° under reduced pressure and oxidized with sodium bismuthate as above to 3β:11β-dihydroxy-2α-methyl-5α-androstan-17-one (XXV). Owing to a faulty sample of bismuthate, 50% of the product was the related 11-ketone (XXVI). This was proved by acetylation of the two products of the reaction and oxidation with chromic acid to obtain identical products. The oxidation product of the ester of the 11β-hydroxyl group gave the rapid pink coloration with alkaline *m*-dinitrobenzene characteristic of 11:17-dioxo steroids. Both products of the original oxidation with bismuthate were oxidized with chromic acid to the same substance which had the properties expected of 2α-methyl-5α-androstane-3:11:17-trione (XXIII).

Methods

The administration of steroids by mouth, collection of urine, extraction, estimation of steroids, chromatography and identification were carried out by the earlier methods. The solvent systems used (Bush & Mahesh, 1959*a*) were: (1) benzene–methanol–water (2:1:1, by vol.); (2) toluene–methanol–water (4:3:1, by vol.); (3*a*) light petroleum (b.p. 100–120°)–benzene–methanol–water (67:33:80:20, by vol.); (3*b*) light petroleum–toluene–methanol–water (67:33:85:15, by vol.); (4*a*) light petroleum–methanol–water (25:24:1, by vol.); (4*b*) light petroleum–methanol–water (20:17:3, by vol.).

EXPERIMENTAL AND RESULTS

Metabolism of cortisone (*Experiment A*, *Table* 1)

A male subject aged 21 years took 5 mg. of prednisone (1:2-dehydrocortisone) by mouth on rising (7.0 a.m.), at noon and at 7.0 p.m. for 2 days. He collected his urine from 9.0 a.m. on the second day to the same time next day and took 100 mg. of

Table 2. *Steroids in plasma after cortisone*

Blood was drawn: 1, 5 min.; 2, 2·08 hr.; 3, 4·08 hr., after ingestion of 100 mg. of cortisone acetate (Expt. *A*).

	Steroids in plasma (μg./100 ml.)		
Sample ...	1	2	3
Cortisol	1·0	15	18
Cortisone	<0·5	?1·0	<1·0

cortisone acetate by mouth at 9.0 a.m. Blood samples were taken at 9.05 a.m., 11.05 a.m. and 1.05 a.m. and plasma was immediately separated by centrifuging. The results are given in Tables 1 and 2.

Identification of metabolites (Chart 1). In view of earlier work, chromatographic identification was carried out by rather fewer degradative reactions than usual. Half of the 24 hr. sample of urine was extracted by procedure 2 of Bush & Mahesh (1959*a*). The extract was pre-fractionated on a 25·4 cm. wide sheet of Whatman no. 3MM paper by a single-length run in system 3*a* and the usual three fractions were eluted. The most polar fraction (no. 3, Bush & Willoughby, 1957) was then run on Whatman no. 3MM in a 25·4 cm. wide lane for 6 hr. in system 1. The main products were detected on strips (3–5 mm.) cut from the main lane by treatment with NaOH, and alkaline blue tetrazolium, and were eluted from the main lane.

Cortisol (I) was identified by its position after a 6 hr. run in system 2 and yellow fluorescence with NaOH, and by its position after a 16 hr. run in system 1 and its reduction of blue tetrazolium. Previous work showed that 95% or more of material in such urine extracts giving a yellow fluorescence in the position of cortisol after a 6 hr. run in system 2 was in fact cortisol, since acetylation gave a homogeneous product with the same R_F as cortisol acetate in systems 2 and 3*a*, and oxidation with bismuthate gave a homogeneous product with the colour fluorescence reactions, and position of 11β-hydroxyandrost-4-ene-3:17-dione (V) in system 3*a*. Similar evidence was obtained with urinary material identified as cortisone (II) via (V) and (VI).

20-Dihydrocortisol (11β:17α:20ξ:21-tetrahydroxypregn-4-en-3-one; III) was identified by its position on chromatograms run for 16 hr. in system 1, the mobility of its acetate in system 3*b*, its yellow fluorescence with NaOH, its failure to reduce blue tetrazolium and its oxidation with periodate to 11β-hydroxyandrost-4-ene-3:17-dione, which was obtained in 95% yield and had the R_F, colour reaction with alkaline *m*-dinitrobenzene, and fluorescence with NaOH characteristic of this substance.

20-Dihydrocortisone (17α:20ξ:21-trihydroxypregn-4-ene-3:11-dione; IV) was identified similarly, except that, on oxidation with periodate, androst-4-ene-3:11:17-trione (VI) was obtained.

One-hundredth of the 24 hr. sample was run in system 1 for 16 hr. and the zone from the origin to 2 cm. behind the 'tail' of tetrahydrocortisol eluted. This was then oxidized with sodium bismuthate and the products were estimated by scanning after chromatography in system 3*b* for 14 hr. There were found 11-oxoaetiocholanolone (25 μg.), 11β-hydroxyaetiocholanolone (13 μg.) and 11β-hydroxyandrosterone (8 μg.). After correction for recovery and differences in molecular weight, this gave the 24 hr. excretion rates: cortol + β-cortol, 4 mg.; cortolone + β-cortolone, 6·1 mg.; *allo*cortol (Bush & Mahesh, 1959*a*) [+probably *allo*-β-cortol (Fukushima *et al.* 1955)], 2·4 mg.

The other metabolites were identified by their positions and colour reactions, coupled with absence of fluorescence with NaOH, on the routine chromatograms used for the quantitative estimations. Previous work (De Courcy, Bush, Gray & Lunnon, 1953; Bush & Willoughby, 1957; Bush & Mahesh, 1959*a*) and the considerable amount of

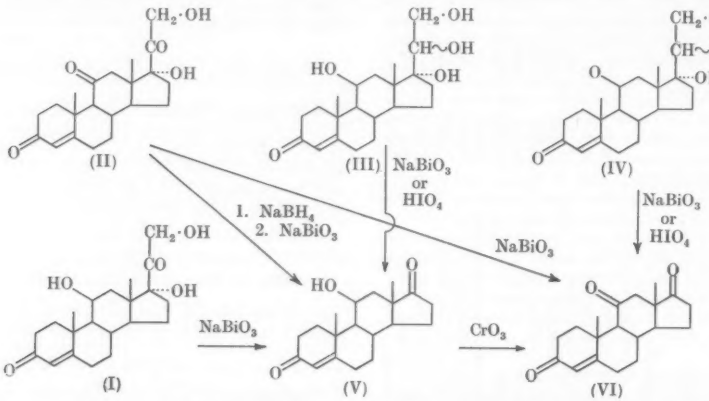


Chart 1. Degradations used to characterize metabolites of cortisone (II) and cortisol (I). (I), (II), (III), (IV) and their acetates, and (V) and (VI) were available as reference substances.

information available on these metabolites (Lieberman & Teich, 1953; Roberts & Szego, 1955) are believed to justify these identifications.

Estimation of plasma steroids. Plasma (5–20 ml.) was diluted with 1 vol. of water and 0·5 vol. of N-NaOH and the solution extracted twice with 3 vol. of ether–ethyl acetate (2:1, v/v). The combined extracts were washed once with 0·05 vol. of 0·1N-NaOH and twice with 0·05 vol. of water. After adding 2–3 drops of acetic acid and filtering through 4–5 g. of anhydrous sodium sulphate, the extract was evaporated at 40° (or below) under reduced pressure with a very fine capillary leak. It was then deposited on a 5 cm. wide strip of Whatman no. 2 paper and run up to the origin in the usual way. The 'band' at the origin thus obtained was then concentrated to a 'spot' 3–4 mm. diam. by 'running in' with ethyl acetate–methanol (2:1, v/v) delivered to the sides of the strip with a syringe-operated pipette. The chromatogram was then run in system 2 for 4 hr. after equilibration for 3 hr. and finally examined with the NaOH fluorescence reaction (Bush, 1952; Bush & Mahesh, 1959*b*). At the time of these experiments, recovery was checked with each batch of plasma by adding three different amounts of cortisol (5–10 μg./100 ml.) to water and extracting 5–10 ml. volumes by the above method; this was because of a period of difficulty with solvents leading to low recoveries (50–60 % instead of the usual 85–95 % at plasma concentrations of 5–10 μg./100 ml.). The results obtained, corrected for the 70 % recovery with this batch of extractions, are given in Table 2. Identification is based solely on chromatographic mobility and NaOH fluorescence reaction.

Metabolism of 2α-methylcortisone (Experiment B, Tables 1 and 3)

A male subject aged 29 years collected a 24 hr. sample of his urine starting at 9.0 a.m. and took 50 mg. of 2α-methylcortisone by mouth at 9.0 a.m. Blood samples were taken at 12.20 p.m. and 3.15 p.m. and the plasma was separated at once. A control sample of urine was collected over the preceding 24 hr.

Preliminary extraction and estimation. One-twentieth of the second urine sample was subjected to extraction procedure 2 (Bush & Mahesh, 1959*a*) and the metabolites of cortisol were estimated as usual. Preliminary estimations of the metabolites of 2α-methylcortisone were also made where their chromatographic positions were characteristic. The very polar metabolites, with mobilities similar to or less than that of cortisol, were estimated by the NaOH fluorescence reaction by visual comparison with 2α-methylcortisol as standard, and given numbers for further identification. Subsequently these metabolites were estimated again, after their structures had been determined, by oxidation with bismuthate to the related 17-ketones and estimating the latter by the scanning method after chromatography and treatment with alkaline *m*-dinitrobenzene (Bush & Mahesh, 1959*a*). The latter estimations are used in Table 3 and are described in full below.

Identification of metabolites. Half (770 ml.) of the urine sample collected during the second day was extracted by procedure 2 (Bush & Mahesh, 1959*a*) by using 75 ml. of resin in chloride form, packed under gravity in a column 2·5 cm. × 15 cm. The urine was percolated at 5 ml./min. and was followed by 100 ml. of glass-distilled water at the same rate. The column was eluted with 500 ml. of methanol–ammonia (sp.gr. 0·880) (19:1, v/v), which was probably excessive in view of later experience. The methanol–ammonia was evaporated under reduced pressure at room temp. (approx. 19°) for 15 min. and then to near dryness at 40–45°. The residue was dissolved in 20 ml. of water and extracted twice with 60 ml. of ether–ethyl acetate (2:1, v/v). The combined extracts were then washed thrice with 6 ml. of N-NaOH and once with 6 ml. of water. The washed extract was partially dried by filtering slowly through 0·5 g. of anhydrous Na_2SO_4 acidified with two drops of acetic acid, and evaporated to dryness under reduced pressure at 40–45°. This extract was called MEU. The aqueous residues were combined and brought to pH 4·8 and 0·5M-acetate by adding 5M-acetic acid and 0·5M-acetic acid–sodium acetate buffer, to reach a final volume of 60 ml. After adding 2·5 ml. of succus entericus of *Helix pomatia* [approx. 200 000 Fishman units of β-glucuronidase, Talalay, Fishman & Huggins (1946)] the solution was incubated for 18 hr. at 45°. The solution was then extracted twice with 180 ml. of ether–ethyl acetate (2:1, v/v) and the extract washed thrice with 18 ml. of N-NaOH and once with 18 ml. of water. It was then dried, acidified, and evaporated as above. This extract was called MEH.

Prefractionation. Extract MEU was deposited over the whole width, 25·4 cm., of a sheet of Whatman no. 3MM paper, and extract MEH over two similar sheets. Small parts of each extract were run similarly on smaller sheets of 3MM paper in parallel with known reference steroids. The sheets were run for 1·8 hr. in system 3*a* after 3 hr. equilibration and three fractions eluted as usual (Bush & Willoughby, 1957): fraction 1 contained the least polar steroids, fraction 2 those of medium polarity such as 11β-hydroxyandrosterone (3α:11β-dihydroxy-5α-androstan-17-one), and fraction 3 the polar steroids such as cortisone and its C_{21} metabolites.

A small part of each fraction was then run in an appropriate system and the chromatograms were examined with the NaOH fluorescence reaction, the blue-tetrazolium reaction, and alkaline *m*-dinitrobenzene. This fractionation is shown in Fig. 1 together with the solvent systems, times of running and types of paper used. With these chromatograms as models, the whole of each fraction was run on a 25·4 cm. wide sheet of no. 3MM paper and fractions were cut and eluted as shown superimposed upon the chromatograms of Fig. 1, with the same solvent systems and other conditions. The positions for cutting off the fractions were determined by inspection of the chromatograms under short-wave light (2537 Å; Chromatolite, Hanovia Ltd., Slough, Bucks). Minor components could not be detected with certainty but were located by using the positions of various fluorescent bands whose relation to the steroids had been determined previously on the preliminary chromatograms of small parts of each fraction. Similar fractions from MEU and MEH were combined for identification.

Fraction 3 (most polar) was run first in system 2 for 3 hr. (Fig. 1). The most polar region of this sheet was then eluted and the subfraction obtained run again in system 1 for 8 hr. One chromatogram each was found sufficient for fractions 1 and 2 and no. 2 paper was used since these fractions were quite clean.

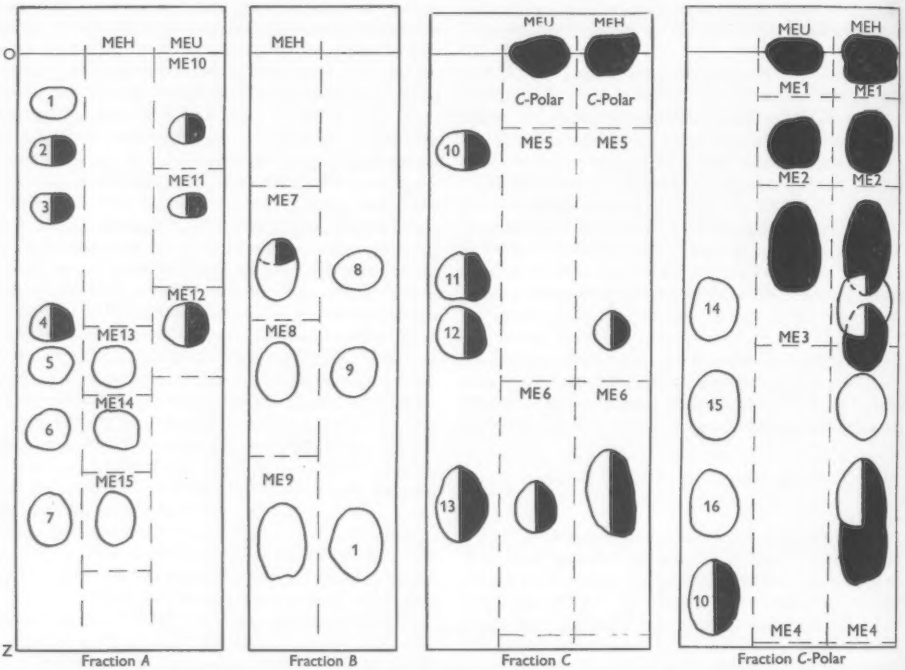


Fig. 1. Second prefractionation of urine extracts from subject who ingested 2α-methylcortisone (semi-diagrammatic). Fractions taken from a first prefractionation as in Bush & Willoughby (1957). ME7–ME15 are those subfractions described in full in the experimental section. O, Origin; Z, solvent front. Fraction *A* (non-polar steroids; Bush & Willoughby, 1957). 1, 11-Oxoaetiocholanolone; 2, adrenosterone; 3, 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione; 4, 2α-methyladrenosterone; 5, dehydro*epi*androsterone; 6, aetiocholanolone; 7, androsterone. Run in system 4*b*, 16 hr. MEU, free steroids; MEH, steroids extracted after enzymic hydrolysis. ○, Colour with alkaline *m*-dinitrobenzene; ●, yellow fluorescence with NaOH. Fraction *B* (medium polarity). 8, 11β-Hydroxyaetiocholanolone; 9, 11β-hydroxyandrosterone. Run in system 3*b* for 14 hr. ○, Colour with alkaline *m*-dinitrobenzene; ●, yellow fluorescence with NaOH. Fraction *C* (polar). 10, Cortisol; 11, cortisone; 12, 2α-methylcortisol; 13, 2α-methylcortisone. Run in system 2 for 3 hr. on Whatman no. 3MM paper. Fraction *C*-polar ('*C*-polar' from previous chromatogram). 14, Tetrahydrocortisol; 15, *allo*tetrahydrocortisol; 16, tetrahydrocortisone. Run in system 1 for 8 hr. on Whatman no. 3MM paper. ○, Reducing blue tetrazolium; ●, yellow fluorescence with NaOH.

Each subfraction as numbered in Fig. 1 was then subjected to the sequential degradation procedures described in detail by Bush & Willoughby (1957) and Bush & Mahesh (1959*a*). As expected, many of these subfractions contained a mixture of products. As these were revealed by successive degradations, the derivatives were eluted separately from the chromatograms so that further degradations were carried out with pure fractions. It should be emphasized that each degradation was carried out with a known reference substance, containing the group altered by the reactions, in parallel with the urinary fraction under examination, and the yield of major and minor reaction products carefully compared. To avoid misidentifications, the quantities used in these degradations were such that 15–30 μg. of each major product was obtained: minor products or major products from impurities can usually be detected with the Zimmermann reaction or with the NaOH fluorescence reaction, in amounts of 0·2–0·5 μg. according to the exact nature of the impurity or minor product. In this way it is easy to decide whether a degradation product is that expected from the material in the reaction concerned or whether it must be considered a derivative of an impurity (Bush & Willoughby, 1957) in the original material.

*Subfraction ME*1 (Chart 2). This material was extremely polar (Fig. 1) and on acetylation gave two products having R_F values of 0·09 (X) and 0·24 (IX) in system 3*a*, the latter predominating, suggesting the presence of at least two esterifiable hydroxyl groups and probably three. These compounds gave a yellow fluorescence with NaOH but no reduction of blue tetrazolium.

Oxidation of the original material with bismuthate or periodate gave two 17-ketones which moved 0·4 and 4·7 cm. when run in system 3*a* for 5·25 hr. The latter (XI) predominated and gave a rapidly developing pink–purple colour with alkaline *m*-dinitrobenzene; the former (XII) gave a

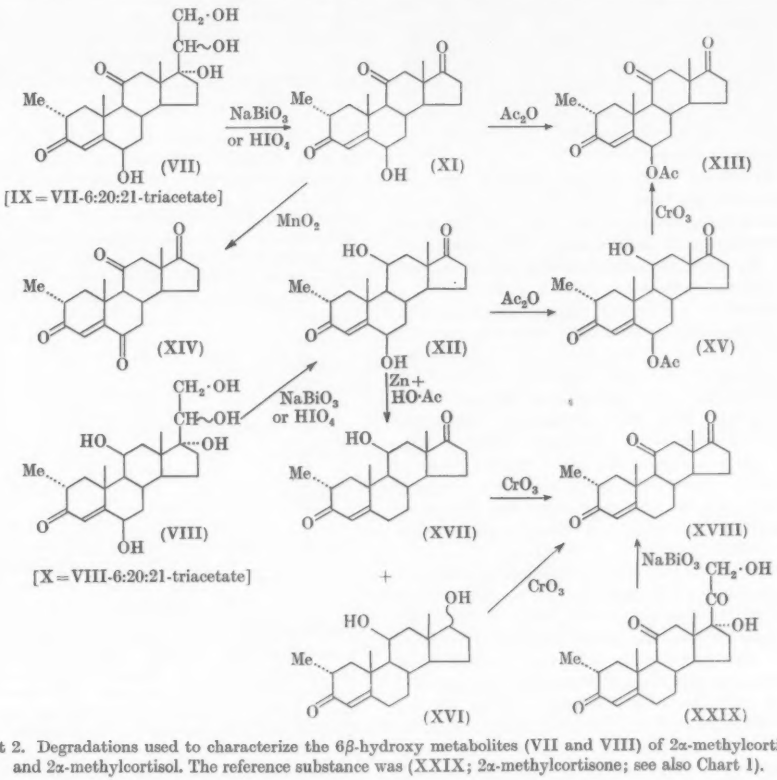


Chart 2. Degradations used to characterize the 6β-hydroxy metabolites (VII and VIII) of 2α-methylcortisone and 2α-methylcortisol. The reference substance was (XXIX; 2α-methylcortisone; see also Chart 1).

slowly developing violet colour. Both gave a fluorescence with NaOH. About 30 μg. of the main bismuthate oxidation product was isolated from another chromatogram after location with ultraviolet light (2537 Å) and dissolved in ethanolic 0·066 N-KOH (3 ml.). The ultraviolet spectrum was measured in the Unicam spectrophotometer (SP. 500) 4 and 24 hr. after mixing, and finally after heating to 60° for 2 hr. The spectra were those characteristic of a 6β-hydroxy-Δ^4-3-oxo steroid (Meyer, 1955) and are given in Fig. 2.

Acetylation of the 17-ketone (XI) gave a new product (XIII) with an R_F of 0·09 in system 3*b*, suggesting the presence of a weakly polar hydroxyl group in the nucleus. Oxidation of (XI) with chromic acid, however, gave some (XIV) and a mixture of products which stayed on or near the origin in system 3*a*, suggesting that ring-opening had occurred. Oxidation of (XIV) with chromic acid for 1 hr. only, however, produced no change in properties, suggesting that the nucleus contained no remaining hydroxyl groups. Oxidation of the acetate (XV) of the minor 17-ketone (XII) yielded a new substance having the same R_F as the acetate (XIII) of the major 17-ketone (XI) and showing the same rapid development of a pink colour with alkaline *m*-dinitrobenzene. This suggested that the minor product (XII) was in fact the 11β-hydroxy analogue of the major product (XI), which was probably an 11-ketone.

The 17-ketone (XI; 50 μg.) isolated from a chromatogram was then oxidized with MnO_2 (1 g.) in refluxing chloroform (50 ml.) for 16 hr. (Amendolla, Rosenkranz & Sondheimer, 1954), the oxide filtered off, and the solution evaporated under reduced pressure at 40–45°. A new product (XIV) was obtained in good yield with an R_F of 0·32 in system 3*b*, and giving an orange colour in the cold with NaOH before giving the yellow fluorescence after heating to dryness (Savard, 1954). This oxidation product was isolated from the rest of the chromatogram and the ultraviolet spectrum examined in ethanol, in ethanolic 0·066 N-KOH, and in the latter solution after neutralizing to pH 6–7 with dilute HCl and finally after bringing to pH 2–3 with acid. The spectra are shown in Fig. 3 and are typical of a 6-oxo-Δ^4-3-oxo steroid (Meyer, 1955).

The product (XIV) was unchanged by chromic acid oxidation for 1 hr. and had an R_F compatible with the structure androst-4-ene-3:6:11:17-tetraone. This suggested that the group oxidized by MnO_2 was the one responsible for ring-opening with chromic acid in the bismuthate oxidation product, and was also the only hydroxyl group in the nucleus. It was also found that very little oxidation of (XI) occurred with MnO_2 in the cold. These properties were compatible only with (XI) having the structure 6β-hydroxy-2α-methylandrost-4-ene-3:11:17-trione, but it was felt desirable to obtain unequivocal degradation to a polyketone which was available as a reference substance, since the 2α-methyl group was present only by inference and was an important group, when interpreting the R_F values. Accordingly, 50 μg. of (XII), which was available in larger amounts from MF 1 (below), was refluxed for 2 hr. with zinc dust (0·1 g.) and acetic acid (10 ml.). After filtering out the zinc dust, water (10 ml.) was added and the mixture brought to pH 5–6 with N-NaOH and extracted twice with 3 vol. of ether–ethyl acetate mixture. The extract was dried over Na_2SO_4 and evaporated under reduced pressure. The extract was run for 3·5 hr. in system 3*b* and, apart from unchanged starting material, contained two substances, one giving a

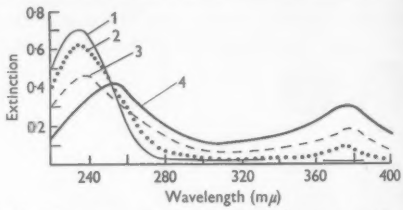


Fig. 2. Ultraviolet spectra of (XI) (bismuthate oxidation product of main component of ME1) (1) (approx. 10 μg./ml.) in ethanol; (2) in ethanolic 0·066 N-KOH after 4 hr.; (3) after 24 hr.; (4) after 24 hr. at room temperature, followed by 2 hr. at 60°. These spectra are characteristic of a Δ^4-6β-hydroxy-3-oxo steroid, and differ from those of 2α-, 6α-, 7α-, 11β-, 11α-, and Δ^4-17β-hydroxy-3-oxo steroids (Meyer, 1955). Similar spectra were obtained with (XXXI) and (XXXIV) (Chart 5).

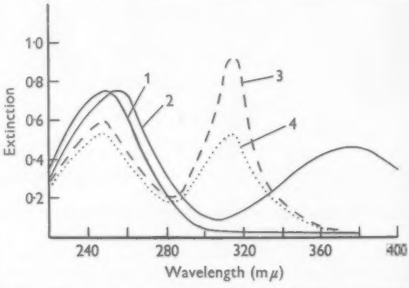


Fig. 3. Ultraviolet spectra of (XIV) (bismuthate oxidation product of ME1, oxidized with manganese dioxide) (1) in ethanol (approx. 10 μg. of steroid/ml.); (2) in ethanolic 0·066 N-KOH; (3) after subsequent acidification to pH 6–7; (4) after further acidification to pH 2–3. These spectra are characteristic of Δ^4-3:6-dioxo steroids (Meyer, 1955). Similar spectra were obtained with (XIV) derived from (XXXI) and (XXXIV) (Chart 5).

yellow fluorescence with NaOH but no colour with alkaline *m*-dinitrobenzene, (presumably XVI), and the other (XVII) giving both reactions. The latter had an R_F identical with that of 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione (0·46 in system 3*b*). Both substances were oxidized with chromic acid to substances giving both reactions, and having R_F values identical with that of 2α-methylandrost-4-ene-3:11:17-trione (XVIII). This degradation to a polyketone with chromatographic properties identical with those of a known reference steroid via a reaction known to be more or less specific for allylic hydroxyl groups (Fieser, 1953) was regarded as adequate proof that the structure of (XI) was 6β-hydroxy-2α-methylandrost-4-ene-3:11:17-trione, and that of (XII) 6β:11β-dihydroxy-2α-methylandrost-4-ene-3:17-dione. The properties of the original material in these fractions were then compatible only with the structures 6β:17α:20ξ:21-tetrahydroxy-2α-methylpregn-4-ene-3:11-dione (VII) for the major, and 6β:11β:17α:20ξ:21-pentahydroxy-2α-methylpregn-4-en-3-one (VIII) for the minor, component.

Subfraction ME2. As expected small traces of (VII, Chart 2) were found by bismuthate oxidation to the 6β-hydroxy compound described above. Small amounts of 11β:17α:20ξ:21-tetrahydroxypregn-4-en-3-one (III, Chart 1) were identified by its known position in this region of the chromatogram, and its oxidation with bismuthate or periodate to 11β-hydroxyandrost-4-ene-3:17-dione (V); the latter gave no acetate and was oxidized with chromic acid to androst-4-ene-3:11:17-trione (VI). Small amounts of 3α:17α:20ξ:21-tetrahydroxy-5β-pregnan-11-one and 3α:11β:17α:20ξ:21-pentahydroxy-5α-pregnane were identified by bismuthate and periodate oxidation to 3α-hydroxy-5β-androstane-11:17-dione and 3α:11β-dihydroxy-5α-androstan-17-one. These were chromatographically identical with their reference steroids and in turn identified by chromatography of their acetates, and oxidation with chromic acid to the 5α- and 5β-androstane-3:11:17-triones (Bush & Mahesh, 1959*a*).

Subfraction ME3. Traces of (VII) (Chart 2) and of 11β:17α:20ξ:21-tetrahydroxypregn-4-en-3-one were identified by oxidation with periodate as above, but were not degraded further owing to lack of material. Tetrahydrocortisol was detected with blue tetrazolium and identified by the procedure of Bush & Mahesh (1959*a*). The main component of this fraction (XIX, Chart 3) gave no fluorescence with NaOH and no reduction of blue tetrazolium. It was detected by the production of a new substance (XX), giving rapidly a pink coloration with alkaline *m*-dinitrobenzene, when ME3 was oxidized with bismuthate or periodate. This presumed 17-oxo steroid had an R_F in system 3*b* of 0·40, compared with 0·26 for 3α-hydroxy-5β-androstane-

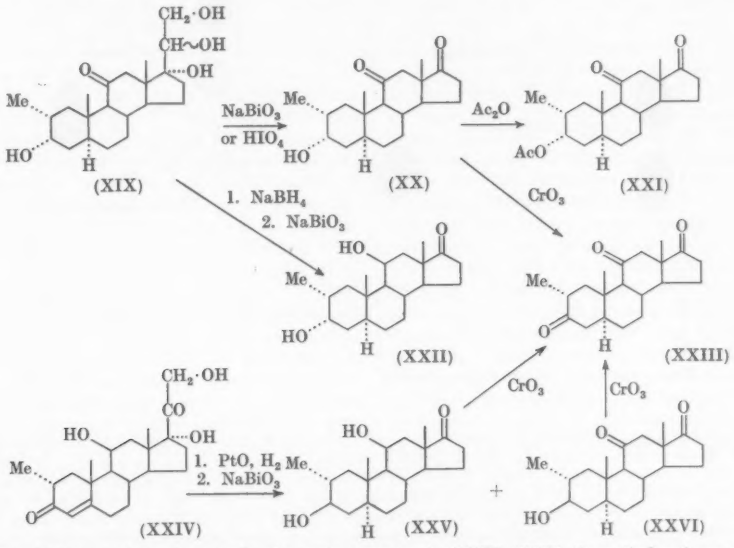


Chart 3. Degradations used to characterize the main component of ME3 (XIX) (2α-methylcortisone experiment). Correlation was achieved by (XXIII) derived via (XXXV) and (XXXVI) from the reference substance (XXIV; 2α-methylcortisol). See text and Fig. 4 for discussion of configuration of 5- H.

11:17-trione (11-oxoaetiocholanolone) and 0·42 for androst-4-ene-3:11:17-trione on the same chromatogram (Fig. 4). Acetylation of the 17-ketone (XX) gave a substance, XXI, with an R_F of 0·61 in system 4*a* compared with 0·56 for 11-oxoaetiocholanolone acetate on the same chromatogram (Fig. 4). The acetate was not altered by oxidation with chromic acid. The original material (XIX; about 20 μg.) was therefore refluxed with sodium borohydride (15 mg.) in tetrahydrofuran (10 ml.) and NaOH (2·57 %, w/v; 1·0 ml.) for 10 hr. Water (10 ml.) was added and the tetrahydrofuran evaporated under reduced pressure. The residue was extracted with ether–ethyl acetate as usual and the reduction product oxidized with sodium bismuthate. A 17-ketone (XXII) was obtained which had an R_F of 0·25 in system 3*b* and gave a slowly-developing violet colour with alkaline *m*-dinitrobenzene. This change in behaviour is characteristic of the reduction of an 11-ketone to an 11β-alcohol (Fig. 4).

Chromic acid oxidation of the 17-ketone (XX) isolated from a chromatogram gave a substance

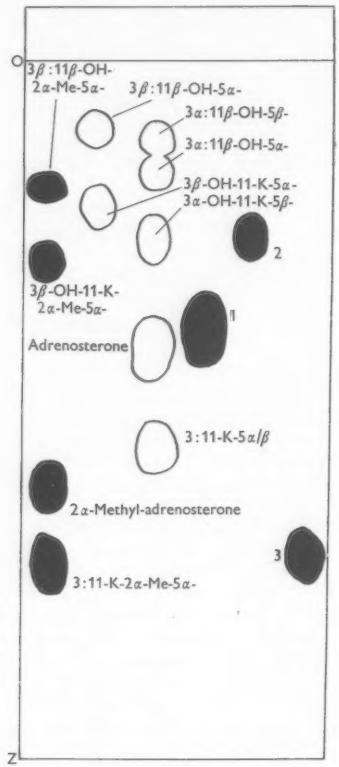


Fig. 4. Chromatographic correlation of degradation products of XIX (Chart 3) (ME3) with saturated 2α-methyl steroids. ●, 2α-Methyl steroids; ○, non-methylated steroids. (*a*) 1, XX; 2, XXII; 3, XXIII. Reference steroids are all derivatives of androstan-17-one, except where full names are given. Run in system 3*b* for approx. 4 hr. O, origin; Z, solvent front. (*b*) 3-Acetates of androstan-17-one derivatives. Run in system 4*a* for approx. 4 hr. 4, Acetate of XX (XXI, suggested as 3α-acetoxy-2α-methyl-5α-androstane-11:17-dione). Note that 5α-H and 5β-H epimers have their relative mobilities inverted on acetylation. In this respect the relationship of 11-oxoaetiocholanolone and its 3-acetate with 1 and 4, and of 3β-hydroxy-2α-methyl-5α-androstane-11:17-dione and its 3-acetate with 1 and 4 strongly suggest the structure 5α-H–3α-OH for 1. Abbreviated nomenclature: Me = methyl; OH = hydroxy or dihydroxy; K = oxo or dioxo.

having an R_F of 0·72 in system 3*b* and giving a bluish violet colour with alkaline *m*-dinitrobenzene. This was identical with 2α-methyl-5α-androstane-3:11:17-trione (XXIII) [the 5α-(H) and 5β-(H)-*cis–trans* epimers of the 3-ketones are not separated in these systems] prepared from 2α-methylcortisol (XXIV) by the route described above (Materials).

The oxidations of the original material in this subfraction are compatible with a side chain having the 17:20-diol or the 17α:20ξ:21-triol structure (XIX). The position of this substance on the original chromatogram is, however, compatible only with the latter structure. The configurations at C-5 and C-3 are discussed below (Discussion).

Subfraction ME 4. This fraction contained traces of 11β:17α:20ξ:21-tetrahydroxy-2α-methylpregn-4-en-3-one (XXVII, Chart 4) and larger quantities of its 11-oxo analogue (XXVIII). It also contained tetrahydrocortisone and *allo*tetrahydrocortisol (3α:11β:17α:21-tetrahydroxy-5α-pregnan-20-one). These were identified by the positions and properties of the original materials, of their acetates (Fig. 5), and by stepwise degradation to the appropriate triones as above, and as in Bush & Mahesh (1959*a*).

Subfraction ME 5. This contained a substance having the mobility of 2α-methylcortisol (XXIV, Chart 4), its reduction of blue tetrazolium, and a rather weak fluorescence with NaOH. About 10 μg. was oxidized with bismuthate and yielded a 17-ketone (XVII), having the R_F, fluorescence with NaOH and colour with alkaline *m*-dinitrobenzene of 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione prepared in parallel from 2α-methylcortisol.

Subfraction ME 6. The main component had the same R_F of 0·80 in system 1 as 2α-methylcortisone (XXIX, Chart 4), reduced blue tetrazolium and gave a weak fluorescence with NaOH. Acetylation gave a substance, with the same reactions, which moved 11 cm. on running for 5·25 hr. in system 3*b*, as did the acetate of 2α-methylcortisone (XXX). Oxidation with bismuthate gave a substance with the same reactions and mobility in system 4*a* as 2α-methylandrost-4-ene-3:11:17-trione (XVIII) (13·8 cm. in 16 hr.). This material was unchanged by acetylation and by chromic acid. After preparation of the ethylene ketal, the material was reduced with sodium borohydride, hydrolysed (Bush & Mahesh, 1959*a*), and oxidized with sodium bismuthate. The product gave a fluorescence with NaOH, a violet colour with alkaline *m*-dinitrobenzene, and moved 9·6 cm. in 16 hr. in system 4*a* (XVII, 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione: 9·6 cm.). The main component was therefore identified as 2α-methylcortisone.

Subfraction ME 7. Two substances were found on chromatograms in system 3*b*. They and their acetates had the properties of 3α:11β-dihydroxy-5β-androstan-17-one and 6β-hydroxy-2α-methylandrost-4-ene-3:11:17-trione (XI, Chart 2) and their acetates. The acetate of the latter was unchanged by chromic acid, but there was too little material to carry the identification to completion. The former was identified by the method of Bush & Mahesh (1959*a*).

Subfraction ME 8. This material behaved like 11β-hydroxyandrosterone and was identified by the procedure of Bush & Mahesh (1959*a*).

Subfraction ME 9. This material was identified as 11-oxoaetiocholanolone by the above methods (as ME 8).

Subfraction ME 10. This material gave a rapid pink colour with alkaline *m*-dinitrobenzene; and both an orange colour in the cold with NaOH and a yellow fluorescence after heating with NaOH. It moved 4·3 cm. in 14 hr. in system 4*b* and was not changed by acetylation or by chromic acid. There was too little material to complete the identification but it seems reasonable, on the basis of the more complete degradation of ME 1, to identify this substance as 2α-methylandrost-4-ene-3:6:11:17-tetraone (XIV, Chart 2).

Subfraction ME 11. This fraction contained a substance giving a fluorescence with NaOH and a violet colour with *m*-dinitrobenzene, and moved 7·9 cm. in 14 hr. in system 4*b*, as did 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione (XVII, Chart 4). It was unchanged by acetylation and on oxidation with chromic acid gave a substance which moved 13·8 cm. in system 4*a* in 16 hr. as did 2α-methylandrost-4-ene-3:11:17-trione (XVIII, Chart 4). This substance was therefore identified as 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione.

Subfraction ME 12. This material gave a fluorescence with NaOH and a rapidly formed pink coloration with alkaline *m*-dinitrobenzene, and moved 13·1 cm. in system 4*b* in 14 hr., as did 2α-methylandrost-4-ene-3:11:17-trione (XVIII). It was unaltered by acetylation or chromic acid. It was therefore identified as the trione.

Subfractions ME 13, *ME* 14 *and ME* 15. These fractions contained respectively 3β-hydroxyandrost-5-en-17-one (DHA), 3α-hydroxy-5β-androstan-17-one (aetiocholanolone) and 3α-hydroxy-5α-androstan-17-one (androsterone). They were identified by their highly characteristic positions after running in systems 4*a* and 4*b* (Bush & Willoughby, 1957), the mobilities of their acetates in system 4*a*, and the resistance of their acetates to alteration by chromic acid.

Examination for unhydrolysed conjugates. In view of the possibility that 3-glucuronosides of the 2α-methyl steroids might not be hydrolysed by β-glucuronidase, the presence of such conjugates was sought by oxidative fission (Norymberski & Sermin, 1953) via the formates.

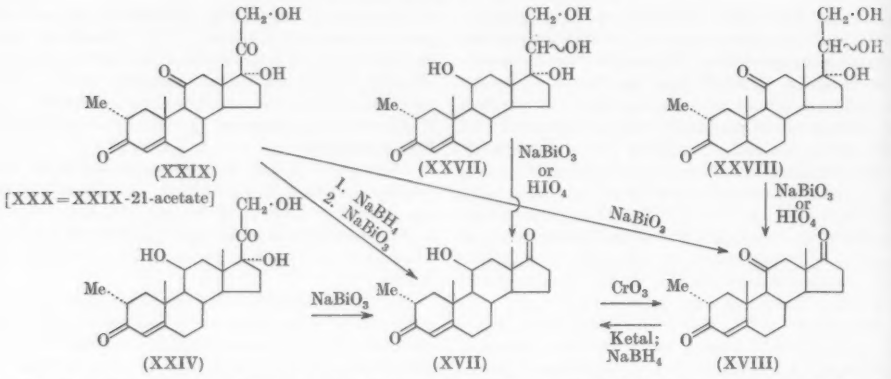


Chart 4. Degradations used to characterize metabolites of 2α-methylcortisone (XXIX) and 2α-methylcortisol (XXIV). Reference substances (XXIX) and (XXIV).

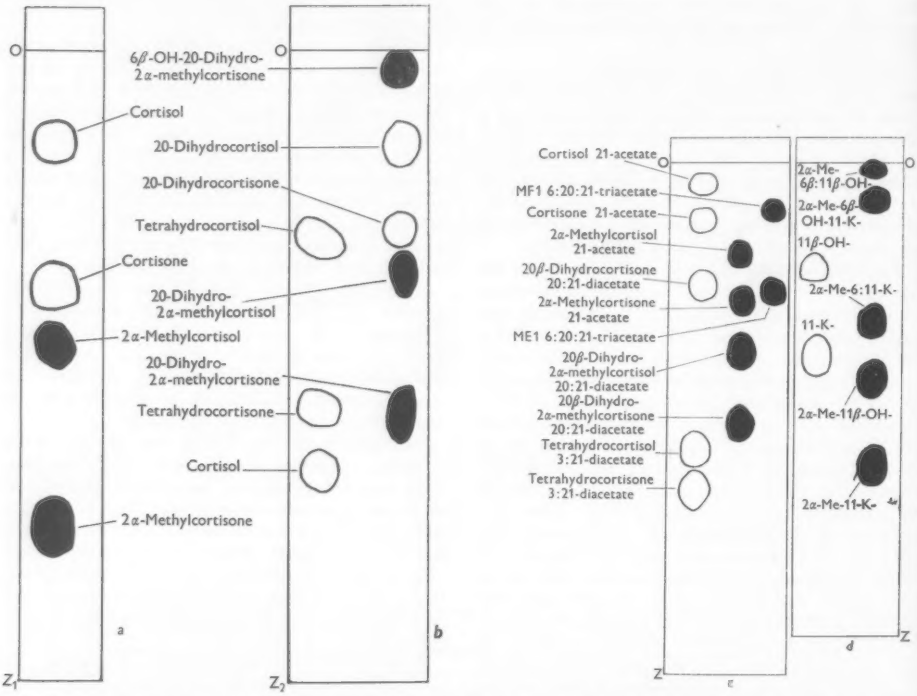


Fig. 5. Chromatographic correlations of 2α-methyl steroids (●) with non-methylated analogues (○) (semi-diagrammatic). O, Origin; Z, bottom of sheet. (*a*) System 1, 3 hr. run on Whatman no. 3 MM paper. (*b*) System 1, 16 hr. run. (Slight taper not shown.) (*c*, *d*) System 3*b*, approx. 3·5 hr. Unless full names are given, the compounds are the derivatives indicated of androst-4-ene-3:17-dione. Other correlations are shown in Fig. 4. Abbreviations as in Fig. 4.

One-twentieth of the 24 hr. urine sample was extracted by the resin method and the extract dissolved in buffer and hydrolysed with succus entericus of *H. pomatia* as usual. The aqueous solution was then extracted with ether–ethyl acetate (2:1, v/v) and the extract washed with N-NaOH and water as usual. The aqueous residue and washes were then combined and brought to pH 3 with HCl and re-extracted by the resin method. The dry extract was oxidized with sodium bismuthate according to the general procedure (Bush & Mahesh, 1959*a*). The oxidation products were dissolved in benzene (10 ml.) and run on a column of alumina (10 g., Savory and Moore, grade II–III). After 24 hr. of contact with the alumina, the column was eluted with ethyl acetate–methanol (1:1, v/v; 100 ml.) and the eluate evaporated to dryness under reduced pressure at 40–45°. It was then run in system 3*b* for 3·5 hr. and the chromatogram treated with alkaline *m*-dinitrobenzene. No 17-oxo steroids were detected, suggesting that less than 0·5 μg. of any single component of this type was present in the oxidized extract. As a control, a concentrate of the glucuronosides of tetrahydrocortisol, *allo*tetrahydrocortisol and tetrahydrocortisone from another urine sample, prepared by the methods of Bush & Gale (1957) and Bush (1957), was treated with bismuthate and alumina

Fig. 6. Scanning records of chromatograms used for estimation of very polar compounds by their oxidation to 17-ketones with bismuthate. Strips run in system 3*b* for 14 hr. and scanned after treatment with alkaline *m*-dinitrobenzene. The extinction was read on an EEL scanner. *A*, Standard reference steroids: 1, 11β-hydroxyaetiocholanolone; 2, 11β-hydroxyandrosterone; 3, 11-oxoaetiocholanolone. *B*, Oxidation products from experiment *B* (2α-methylcortisone metabolites). 4, 6β:11β-dihydroxy-2α-methylandrost-4-ene-3:17-dione derived from (VIII) (Chart 2); 5, 6β-hydroxy-2α-methylandrost-4-ene-3:11:17-trione derived from (VII) (Chart 2); 6, unidentified: probably 11β-hydroxyandrost-4-ene-3:17-dione derived from traces of 20-dihydrocortisol. *C*, Oxidation products from experiment *C* (2α-methylcortisol metabolites). Other details are in experimental section.

Table 3. *Excretion of metabolites of 2α-methylcortisol and 2α-methylcortisone*

Methods of estimation: 1, oxidation with $NaBiO_3$, chromatography of oxidation products, and scanning the chromatogram after treatment with alkaline *m*-dinitrobenzene; 2, NaOH fluorescence, visual matching; 3, scanning of chromatogram after treatment with alkaline *m*-dinitrobenzene. All figures were corrected by assuming the recovery values of the last column. Figures for method 1 were corrected for losses in $NaBiO_3$ oxidation step and for molecular-weight difference due to oxidation.

Metabolites	Method of estimation	Expt. *B* 2α-Methylcortisone (mg.)	Expt. *B* 2α-Methylcortisone (% of dose)	Expt. *C* 2α-Methylcortisol (mg.)	Expt. *C* 2α-Methylcortisol (% of dose)	Fraction no. of major occurrence	Formula no. and (chart)	Recovery value used (%)
6β:11β:17α:20ξ:21-Pentahydroxy-2α-methylpregn-4-en-3-one	1	0·28	0·56	6·8	13·6	MF1	VIII (2)	60
6β:17α:20ξ:21-Tetrahydroxy-2α-methylpregn-4-ene-3:11-dione	1	4·8	9·6	1·2	2·4	ME1	VII (2)	60
11β:17α:20ξ:21-Tetrahydroxy-2α-methylpregn-4-en-3-one	2	0·071	0·14	1·1	2·2	MF3	XXVII (4)	70
17α:20ξ:21-Trihydroxy-2α-methylpregn-4-ene-3:11-dione	2	0·86	1·7	0·071	0·14	ME4	XXVIII (4)	70
11β:17α:21-Trihydroxy-2α-methylpregn-4-ene-3:20-dione (2α-methylcortisol)	2	0·11	0·22	1·7	3·4	MF5	XXIV (4)	70
17α:21-Dihydroxy-2α-methylpregn-4-ene-3:11:20-trione (2α-methylcortisone)	2	1·1	2·2	0·36	0·72	ME5	XXIX (4)	70
3α:17α:20ξ:21-Tetrahydroxy-2α-methyl-5α-pregnan-11-one	1	2·0	4·0	<0·05	—	ME3	XIX (3)	70
6β:11β-Dihydroxy-2α-methylandrost-4-ene-3:17-dione	2	~0·03	0·06	—	—	MF7	XII (2)	80
6β-Hydroxy-2α-methylandrost-4-ene-3:11:17-trione	2	~0·03	0·06	—	—	ME7	XI (2)	80
11β-Hydroxy-2α-methylandrost-4-ene-3:6:17-trione	2	~0·03	0·06	—	—	MF11	—	80
2α-Methylandrost-4-ene-3:6:11:17-tetraone	2	~0·03	0·06	—	—	ME10	XIV (2)	80
11β-Hydroxy-2α-methylandrost-4-ene-3:17-dione	3	0·047	0·09	0·23	0·46	MF12	XVII (4)	85
2α-Methylandrost-4-ene-3:11:17-trione	3	0·29	0·58	0·059	0·12	ME12	XVIII (4)	85
		Total	19·33		23·04			

in the same way and gave an 80–90 % yield of the expected 11β-hydroxyaetiocholanolone, 11β-hydroxyandrosterone and 11-oxoaetiocholanolone. It was concluded that apart from subfraction ME 3 no 3-hydroxy metabolites of 2α-methylcortisone were excreted in significant amounts in the form of glucuronosides.

Quantitative estimation of steroids. After identification of the steroids in this urine sample had been completed, the more polar metabolites were estimated again by oxidation with bismuthate to the 17-oxo steroids and chromatography of the latter. After treatment of the chromatograms with alkaline *m*-dinitrobenzene, they were scanned. 2α-Methylcortisol and 2α-methylcortisone, 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione and 2α-methyladrenosterone were not re-estimated by this method since they had been shown to be homogeneous on the original chromatograms.

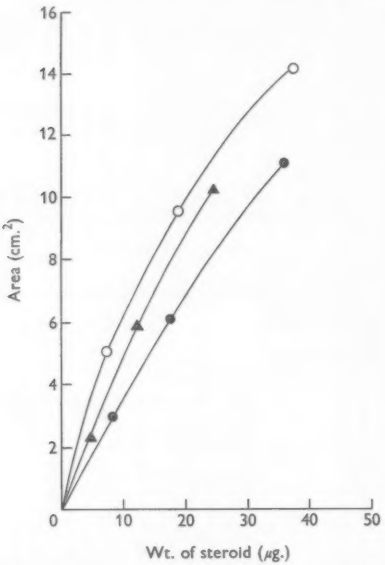


Fig. 7. Calibration curves for 2α-methylsteroids using bismuthate oxidation followed by chromatography of the 17-ketones, and scanning after treatment with alkaline *m*-dinitrobenzene. ○, 11-Oxoaetiocholanolone standard; ▲, 2α-methyladrenosterone from oxidation of 2α-methylcortisone; ●, 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione from oxidation of 2α-methylcortisol. Ordinates: area under peaks measured by the conventions of Bush & Willoughby (1957). Abscissae: μg. of original steroid (i.e. not the bismuthate oxidation product, e.g. the curve for 2α-methyladrenosterone itself would lie above that shown (▲) by an amount determined by the molecular-weight ratio 372/312 and the losses during bismuthate oxidation).

One-hundredth of the 24 hr. sample (15·4 ml.) was extracted by procedure 2 (Bush & Mahesh, 1958*a*) and the extract prefractionated by running for 14 hr. on a 5 cm. wide strip of Whatman 3MM in system 1. The material, more polar than tetrahydrocortisol, was eluted and dissolved in 2 ml. of 50 % (w/v) acetic acid and 50 mg. of sodium bismuthate added. After shaking for 1 hr. the suspension was filtered, treated with sodium metabisulphite (10 %, w/v; 1 ml.) and extracted twice with 3 vol. of ether–ethyl acetate (2:1, v/v). The extract was dried with anhydrous Na_2SO_4 (0·5 g.) and evaporated under reduced pressure at 40–45°. It was then run in system 3*b* for 14 hr. on a 2·5 cm. strip of Whatman no. 2 paper treated with alkaline *m*-dinitrobenzene and scanned. The main products of oxidation were 6β:11β-dihydroxy-2α-methylandrost-4-ene-3:17-dione, 6β-hydroxy-2α-methylandrost-4-ene-3:11:17-trione, 11β-hydroxyandrosterone and 11-oxoaetiocholanolone being derived from VIII, VII (Chart 2), 3α:11β:17α:20ξ:21-pentahydroxy-5α-pregnane and 3α:17α:20ξ:21-tetrahydroxy-5β-pregnan-11-one respectively. In addition, 3α:17α:20ξ:21-tetrahydroxy-2α-methyl-5α-pregnan-11-one gave rise to 3α-hydroxy-2α-methyl-5α-androstane-11:17-dione, which was estimated similarly but with a 14 hr. run in system 4*a*.

The chromatogram of these derivatives is shown in Fig. 6. The results of this estimation are given in Table 3, where they are corrected for both the differences in molar extinctions with alkaline *m*-dinitrobenzene (in the scanning method) and for the differences in the molecular weights of the C_{21} steroids and their bismuthate oxidation products. Calibration curves obtained by the bismuthate oxidation method with 2α-methylcortisol and 2α-methylcortisone are given in Fig. 7.

Distribution of metabolites between MEU and MEH. One-twentyfifth of the 24 hr. sample was extracted with ether–ethyl acetate in the usual way but without enzyme hydrolysis. The aqueous residues were combined, brought to pH 4·8 and 0·5 M concentration of sodium acetate–acetic acid buffer and incubated for 16 hr. at 45°. The extracts of the hydrolysate and the unhydrolysed urine were then run for 6 hr. in system 2 on 2·5 cm. lanes on Whatman no. 2 paper and examined by the NaOH fluorescence method. The results are given in Table 4.

Estimation of steroids in plasma. Plasma was extracted and the Δ^4-3-oxo steroids estimated by the NaOH fluorescence method as above. The results (corrected for 70 % recovery) are given in Table 5.

Table 4. *Distribution of metabolites between free and conjugated forms*

The figures are for one-twentyfifth of the 24 hr. urines. One-twentyfifth of a 24 hr. sample was used for the free steroids, but the figures for conjugated steroids are derived from smaller fractions with which more accurate estimates were possible. Expt. *B*, 2α-methylcortisone as precursor. Expt. *C*, 2α-methylcortisol as precursor. The 6β- and 20ξ-hydroxy metabolites were just detectable in the free steroid fraction; hence the approximation for this figure.

	Free (μg.)		Conjugated (μg.)	
Experiment	*B*	*C*	*B*	*C*
Metabolite				
2α-Methylcortisone	4·3	0	53	11
2α-Methylcortisol	0	8·6	5·0	86
6β-Hydroxy-2α-methylsteroids and 20ξ-Hydroxy-2α-methylsteroids	Approx. 8·0	Approx. 8·0	240	370

Table 5. *Steroids in plasma after 2α-methylcortisone*

Blood was drawn: 1, 3·33 hr.; 2, 6·25 hr. after ingestion of 50 mg. of 2α-methylcortisone (Expt. *B*).

	Steroids in plasma (μg./100 ml.)	
Sample	1	2
Steroid		
Cortisol	0·4	0·4
2α-Methylcortisol	1·5	<0·6
2α-Methylcortisone	15	<1·2

Table 6. *Steroids in plasma after 2α-methylcortisol*

Blood drawn: 1, 2·1 hr.; 2, 4·75 hr. after ingestion of 50 mg. of 2α-methylcortisol (Expt. *C*).

	Steroids in plasma (μg./100 ml.)	
Sample	1	2
Steroid		
Cortisol	<0·2	<0·2
2α-Methylcortisol	5·9	37·5
2α-Methylcortisone	<1·0	<1·0

Metabolism of 2α-methylcortisol (Experiment C, Tables 1 and 3)

A male subject aged 24 years collected two 24 hr. samples of urine beginning at 9.0 a.m. and took 50 mg. of 2α-methylcortisol by mouth at 9.0 a.m. on the second day. Blood samples were drawn at 11.06 a.m. and 1.45 p.m. on the second day. The preliminary extraction and estimation was carried out as with Expt. *B* (above).

Identification of metabolites. Half (520 ml.) of the second urine sample was extracted and fractionated as in Expt. *B* (Fig. 1). Each subfraction (MF) obtained contained the same metabolites as in Expt. *B* but in this experiment the 11β-hydroxyl member of each pair was excreted in much greater amounts than the 11-ketone. The identification of these metabolites was carried out by the same routes of degradation as were used for comparable subfractions (ME) in Expt. *B* except that the preponderant 11β-alcohols were used for carrying out the more extensive degradations to known polyketones. In the case of 6β:11β:17α:20ξ:21-pentahydroxy-2α-methylpregn-4-en-3-one, however, elimination of the 6β-hydroxyl group with zinc and acetic acid was carried out before oxidation of the 11β-hydroxyl group, in order to avoid a mixture of 11α- and 11β-ols. Neither (XIX) (ME 3) nor its 11β-hydroxyl analogue was found in this urine. These degradations are summarized in Charts 2 and 4.

Quantitative estimation. As in Expt. *B*, preliminary estimation was followed by oxidation of the main polar metabolites with sodium bismuthate and estimation of the derived 17-oxo steroids by scanning a chromatogram of these steroids after treatment with alkaline *m*-dinitrobenzene. The scanning trace of this chromatogram is shown in Fig. 6 and the results of these estimations are given in Table 3.

Examintion of unhydrolysed conjugates. This was carried out by the same method as in Expt. *B* with the same negative results.

Distribution of metabolites between free and conjugated fractions. Extracts of untreated urine and of enzymic hydrolysates of the aqueous residues were made as in Expt. *B*, and the steroid content estimated similarly with the NaOH fluorescence reaction. The results are given in Table 9.

Plasma steroids. These were estimated as above on 20 ml. samples. The results are given in Table 6.

Metabolism of adrenosterone (androst-4-ene-3:11:17-trione) (Experiment D, Table 1)

A male subject aged 33 years took 5 mg. of prednisolone by mouth at approximately 8.0 a.m. and 8.0 p.m. for 3 days. On the second day he collected a 24 hr. specimen of urine ending at 10.30 a.m. on the third day. On the third day he took in addition 50 mg. of adrenosterone at 10.30 a.m. and collected a second 24 hr. specimen of urine. At 1.30 p.m. a sample of antecubital venous blood was drawn from which 25 ml. of plasma was separated.

Estimation of steroids in urine. The routine method was applied (Methods) with the results shown in Table 1. In addition, the toluene layer from the urine bottle was separated and evaporated under reduced pressure at 40–45°, after which the residue was run in system 3*b* as for fraction 2 of the usual prefractionation chromatogram. No detectable 17-oxo steroids were found; with the limits of sensitivity of the reaction with alkaline *m*-dinitrobenzene, this means that the toluene layer contained less than 0·05 mg. of any individual 17-oxo steroid (cf. Expt. *E*).

Identification of metabolites. The three principal metabolites were identified by elution from a chromatogram in system 3*b* and preparation of the derivatives given by Bush & Mahesh (1958*b*). In each case the derivatives were chromatographically identical with those derived from the known reference compounds.

Estimation of plasma steroids. This was carried out with 25 ml. of plasma separated from a blood sample drawn 3·0 hr. after ingestion of the steroid under test. The method was the same as above except that the chromatogram was run for 3·5 hr. in system 3*b*. The content of 11β-hydroxyandrost-4-ene-3:17-dione and adrenosterone was 9·0 and 1·0 μg./100 ml. (corr.) respectively.

Metabolism of 2α-methyladrenosterone (2α-methylandrost-4-ene-3:11:17-trione) (Experiment E, Table 7)

A male subject aged 25 years took 5 mg. of prednisolone at 8.0 a.m. and 8.0 p.m. by mouth for 4 days. On the fourth day he took in addition 40 mg. of 2α-methyladrenosterone by mouth at 9.0 a.m. Urine was collected from 9.0 a.m. until 9.0 a.m. the next day. At 12 noon blood was drawn from the antecubital vein from which 25 ml. of plasma was separated.

Identification of metabolites. A quarter of the 24 hr. sample was extracted by the resin method (procedure 2, Bush & Mahesh, 1959*a*) and the extract run for 1·8 hr. in system 3*b* on a 25 cm. wide sheet of Whatman no. 3MM paper. Three fractions corresponding to those taken in the routine prefractionation step were eluted with ethyl acetate–methanol and concentrated with a jet of air.

Fraction 3 (polar steroids) was run on a 12·5 cm. wide sheet of Whatman no. 2 paper for 4 hr. in system 1. Strips 3–5 mm. wide were cut from the sheet and treated with alkaline blue tetrazolium, alkaline *m*-dinitrobenzene, and NaOH. Apart from faint bands with the first two reagents, the only compounds found were two with R_F values of 0·81 and 0·91 which gave a yellow fluorescence with NaOH but no colour with alkaline *m*-dinitrobenzene. The areas containing these substances were eluted and called subfractions MA 1 and MA 2 respectively.

Fraction 2 was run as above, but in system 3*b* for 14 hr. Two substances, 15·3 and 26·7 cm. from the origin, were detected which gave a yellow fluorescence with NaOH and weak colours with alkaline *m*-dinitrobenzene. The region behind the first was eluted and called subfraction MA 3; the former substance was eluted as subfraction MA 4, and the latter as MA 5.

Fraction 1 was run for 14 hr. in system 4*a* as above and three substances reacting with alkaline *m*-dinitrobenzene were detected at positions 4·7, 21·3 (aetiocholanolone 21·3 cm. on the same sheet) and 25·2 cm. (androsterone 25·2 cm. on the same sheet) from the origin. These three areas were eluted as subfractions MA 6, MA 7 and MA 8.

The toluene from the urine bottle was evaporated under reduced pressure at 40–45° and the residue run in system 4*a* as above for 14 hr. Two substances, 8·3 and 14·4 cm. from the origin, were detected on this chromatogram which gave both a yellow fluorescence with NaOH and a violet colour with alkaline *m*-dinitrobenzene (11β-hydroxy-2α-methylandrost-4-ene-3:17-dione 8·3 cm., and 2α-methyladrenosterone 14·4 cm. on the same sheet). These substances were eluted as subfractions MA 9 and MA 10.

Subfraction MA 1. Acetylation gave a substance (XXXII, Chart 5), detected by the NaOH fluorescence reaction, which had an R_F of 0·17 in system 4*a*, showing the presence of at least two esterifiable hydroxyl groups. Oxidation of the acetate gave a substance (XXXIII) with an R_F of 0·29 in system 4*a*, showing the presence (in XXXII) of an unesterified hydroxyl group. Oxidation of the original free steroid (XXXI) with chromic acid for 1 hr. at room temp. gave two main products, and material at the origin when run in system 3*b* for 4 hr. The first had an R_F of 0·09 and gave both the NaOH fluorescence reaction and a violet colour with alkaline *m*-dinitrobenzene; the second (XIV) had an R_F of 0·32 and gave both these reactions. In addition, the latter gave an orange colour in the cold with NaOH and had the same R_F as 2α-methylandrost-4-ene-3:6:11:17-tetraone [isolated from urine of Expt. *B* as a degradation product (XIV, Chart 2)]. These properties were compatible with the structure 6β:11β:17ξ-trihydroxy-2α-methylandrost-4-en-3-one (XXXI) for the original substance and incompatible with any other reasonable steroid formula. About 30 μg. of MA 1 was dissolved in ethanolic 0·06 N KOH acid and examined as above (subfraction ME 1, Expt. *B*, Fig. 2). The spectral changes characteristic of a 6β-hydroxy-4-ene-3-oxo steroid were found (Meyer, 1955).

Subfraction MA 2. Acetylation gave a substance (XXXIII) giving the NaOH fluorescence reaction

with an R_F of 0·29 in system 4*a* (same sheet as with MA1 and derivatives thereof). Oxidation of the acetate with chromic acid yielded the material unchanged. Oxidation of the original free steroid with chromic acid yielded the same products (XIV) obtained from similar oxidation of MA1. The original structure was therefore identified as 6β:17ξ-dihydroxy-2α-methylandrost-4-ene-3:11-dione (XXXIV). The main oxidation products from MA1 and MA2 (believed to be XIV) were combined (30 μg.) and dissolved in ethanolic 0·066N-KOH. The ultraviolet spectra showed the changes characteristic of steroid 3:6-dioxo-4-enes (Meyer, 1955) (see Fig. 3).

Subfraction MA3. This fraction gave neither a fluorescence with NaOH nor a colour with alkaline *m*-dinitrobenzene. Oxidation of the material with chromic acid, however, gave a single substance giving rapidly a violet colour with alkaline *m*-dinitrobenzene and having an R_F of 0·75 in system 3*b* [2α-methyl-5α-androstane-3:11:17-trione (XXIII) 0·75 on the same sheet]. A part of the fraction was run in system 3*b* for 14 hr., in parallel with another part which had been previously

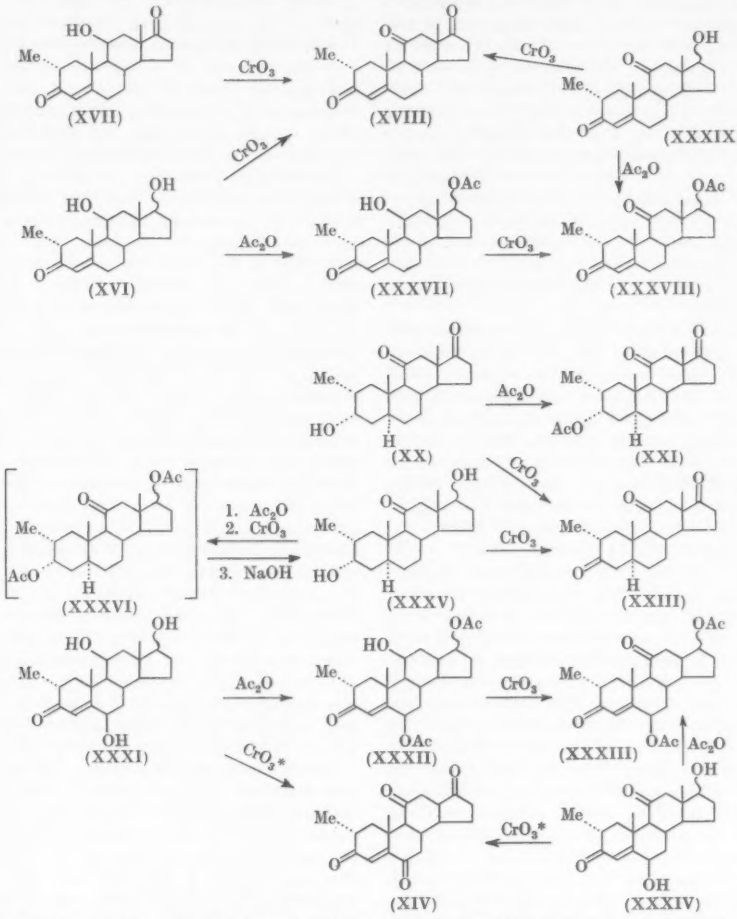


Chart 5. Degradations used to characterize the metabolites of 2α-methyladrenosterone. (XXXVI) was not isolated. Demonstration of the 6β-hydroxyl group is better achieved with MnO_2 oxidation (see XI→XIV, Chart 2). To avoid ring-opening, the oxidations marked (*) were shortened to 1 hr.

acetylated, oxidized with chromic acid and saponified. The chromatogram was dipped in boiling 0·4% chromic acid in 90% acetic acid, dried on a hot plate in an electric oven at 80° and heated with alkaline *m*-dinitrobenzene (Axelrod, 1955). Both fractions gave a bluish pink colour, the spots being 10·1 cm. from the origin. This showed that the oxygen function at C-11 was a ketone group in MA 3. The most probable structure for this substance was 3α:17ξ-dihydroxy-2α-methyl-5α-androstan-11-one (XXXV).

Subfraction MA 4. Acetylation gave a substance (XXXVII) with an R_F of 0·13 in system 4*a*, showing the presence of at least one esterifiable hydroxyl group. Chromic acid oxidation of the acetate gave a new substance (XXXVIII) with an R_F of 0·24 in system 4*a*, showing the presence of an unesterified hydroxyl group. Chromic acid oxidation of the original material gave a substance which moved 13·7 cm. when run in system 4*a* for 16 hr. [2α-methyladrenosterone (XVIII) 13·7 cm. on the same sheet]. Only the last degradation product of MA 4 gave a colour with alkaline *m*-dinitrobenzene, the rapidly developing pinkish purple characteristic of 11:17-dioxo steroids. These properties were compatible only with the structure 11β:17ξ-dihydroxy-2α-methylandrost-4-en-3-one for MA 4 (XVI).

Subfraction MA 5. Acetylation gave a new substance (XXXVIII) with an R_F of 0·24 in system 4*a*. The acetate was unchanged on treating with chromic acid. Oxidation of the original material with chromic acid gave 2α-methyladrenosterone (XVIII), which moved 13·7 cm. when run in system 4*a* for 16 hr. (same sheet as for the oxidation product of MA 4 above) and gave a pink–purple colour with alkaline *m*-dinitrobenzene. The original steroid was therefore identified as 17ξ-hydroxy-2α-methylandrost-4-ene-3:11-dione (XXXIX).

Subfraction MA 6. This material gave a pink–purple colour with alkaline *m*-dinitrobenzene, but no fluorescence with NaOH. It had the same R_F as the bismuthate oxidation product of the main component of subfraction ME 3 (Expt. *B*) and was therefore suspected to be 3α-hydroxy-2α-methyl-5α-androstane-11:17-dione (XX). Acetylation gave a substance with an R_F of 0·61 in system 4*a*, identical with that of presumed 3α-acetoxy-2α-methyl-5α-androstane-11:17-dione (XXI) on the same sheet. Oxidation of the original material with chromic acid, however, gave a substance with an R_F of 0·75 in system 3*b*, identical with that of 2α-methyl-5α-androstane-3:11:17-trione (XXIII) run on the same sheet. The original material was therefore identified as 3α-hydroxy-2α-methyl-5α-androstane-11:17-dione (see Discussion).

Subfraction MA 7. This was identified as aetiocholanolone by its position on the chromatograms run in system 4*a* for 16 hr., the symmetry of the peak obtained on scanning the strip and chromatographic properties of its acetate (Bush & Willoughby, 1957).

Subfraction MA 8. This was identified as androsterone by the same criteria as used for MA 7.

Subfraction MA 9. This material had the R_F, fluorescence with NaOH, and colour with alkaline *m*-dinitrobenzene of 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione (XVII). Acetylation left the material unchanged. On oxidation with chromic acid a single substance was obtained which moved 13·7 cm. when run in system 4*a* for 16 hr. as did 2α-methyladrenosterone (XVIII) on the same sheet. The material was therefore identified as 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione.

Subfraction MA 10. This material had the chromatographic mobility and above two reactions characteristic of 2α-methyladrenosterone (XVIII). Since it was unchanged by acetylation or by chromic acid, it was identified as this substance.

Quantitative estimation of urinary metabolites. None of the metabolites interfered with the routine method of estimating the metabolites of endogeneously produced steroids and these were estimated in the usual way (Bush & Willoughby, 1957; Bush & Mahesh, 1959*a*). Compounds MA 1 and MA 2 were estimated visually with 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione as standard by using the NaOH fluorescence reaction (95% confidence limits approximately ± 15% in the range used). Compounds MA 3, MA 4, and MA 5 were estimated by scanning the chromatogram of their chromic acid oxidation products, after treatment with alkaline *m*-dinitrobenzene and using 2α-methyladrenosterone as standard to allow for the enhancement of extinction due to the 11-oxo group. [The Δ^4-3-oxo group appears to make very little contribution to this colour reaction in the 2α-methyl compounds, whereas the 11-oxo group has its usual effect (Wilson, 1954; fig. 7).] Compounds MA 6, MA 9 and MA 10 were estimated by direct scanning of chromatograms run in system 4*a* for 16 hr. and treated by the standard method with alkaline *m*-dinitrobenzene. 2α-Methyladrenosterone was used as standard for the two 11-ketones, and 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione for MA 9. The results are given in Table 7.

Estimation of plasma steriods. Plasma (12·5 ml.) was extracted with ether–ethyl acetate (Bush & Mahesh, 1959*a*) and the extract run in system 3*b* for 4 hr. by the method of Bush & Mahesh (1959*b*) to concentrate the extract at the origin of the chromatogram in a spot approximately 2 mm. in diameter. The steroids were estimated visually using the NaOH fluorescence reaction and 2α-methyladrenosterone and the related 11β-ol as standards. These two compounds were estimated to

Table 7. *Excretion of metabolites of 40 mg. of 2α-methyladrenosterone (Experiment E)*

Methods of estimation: 1, scanning after treatment of chromatogram with alkaline *m*-dinitrobenzene; 2, NaOH fluorescence; 3, separation on chromatograms, oxidation with CrO_3, chromatography of products and then method 1.

Metabolites	Excretion (mg.)	Excretion (% of dose)	Method of estimation	Fraction no.	Formula no. (Chart 5)	Recovery value assumed (%)
6β:11β:17ξ-Trihydroxy-2α-methylandrost-4-en-3-one	1·0	2·5	2	MA1	XXXI	80
6β:17ξ-Dihydroxy-2α-methylandrost-4-ene-3:11-dione	1·5	3·7	2	MA2	XXXIV	80
11β-Hydroxy-2α-methylandrost-4-ene-3:17-dione	2·0	5·0	1	MA9	XVII	85
2α-Methylandrost-4-ene-3:11:17-trione (2α-methyladrenosterone)	2·8	7·0	1	MA10	XVIII	85
11β:17ξ-Dihydroxy-2α-methylandrost-4-en-3-one	2·4	6·0	3	MA4	XVI	80
17ξ-Hydroxy-2α-methylandrost-4-ene-3:11-dione	4·1	10·0	3	MA5	XXXIX	80
3α:17ξ-Dihydroxy-2α-methyl-5α-androstan-11-one	1·0	2·5	3	MA3	XXXV	80
3α-Hydroxy-2α-methyl-5α-androstane-11:17-dione	0·5	1·3	1	MA6	XX	80
Total	15·3	38·0				

be present in amounts (uncorrected) of 0·5 μg., which is close to the limit of detection and therefore subject to considerable error (±30%). After correction for 80% recovery, this is equivalent to a plasma concentration of 5 μg./100 ml.

DISCUSSION

Reliability of methods of identification and estimation

Chromatographic methods of identifying steroids are still controversial since the number and variety of structures based on the steroid nucleus is enormous and numerous possibilities of confusion exist in the identification of any given steroid. However, our method of stepwise degradation in parallel with known reference steroids is believed to overcome these difficulties and the arguments supporting identification by this method have been given fully in previous papers (Bush, 1954; Bush & Willoughby, 1957; Bush & Mahesh, 1959*a*). Only a few points therefore need attention in the experiments reported here.

The effects of substituents at carbon atoms 3, 11, 17, 20 and 21 were shown to be entirely similar in the 2α-methyl steroids to those found with steroids unsubstituted at C-2 by preparation of derivatives of 2α-methylcortisol by unequivocal reactions. The influence of the 2α-methyl group was the expected one (Martin, 1950) of reducing the polarity of the steroid in which it was a substituent, the effect being similar in the Δ^4-3-ketones and in the saturated 3-ketones. The effect of this group in the 3β-alcohols was, however, rather less than in the 3-ketones. These results are summarized in the chromatograms shown in Figs. 4 and 5.

Identification of the Δ^4-2α-methyl-3-ketones described in this paper is therefore considered to be established beyond reasonable doubt. After the estensive investigations of the structure of 6β-hydroxy-2α-methylandrost-4-ene-3:11:17-trione derived from the bismuthate oxidation of ME1 and MF2 (Expts. *B* and *C*) it was thought sufficient to demonstrate the structures of MA1 and MA2 (Expt. *E*) by carrying their degradation as far as the fully oxidized product 2α-methylandrost-4-ene-3:6:11:17-tetraone (XIV) which had the correct mobility and also gave the characteristic orange colour in the cold with NaOH (Savard, 1954) and spectral changes in ethanolic KOH (Meyer, 1955). A further characteristic was the production of more polar oxidation products, probably with ring A opened, during the oxidation of the related 6β-alcohol with chromic acid. This effect was reduced by shortening the period of oxidation to 1 hr.

Identification of the saturated 3-hydroxy metabolites of these 2α-methyl steroids is, however, still doubtful as regards the configuration at position 5. Inferences based on the characteristic

behaviour of such isomers and their acetates in the series of non-methylated steroids (Bush & Willoughby, 1957) cannot be used with certainty, although they are reasonable, until a complete set of isomers of the 2α-methyl steroids has been prepared. The correlation of these derivatives is summarized in the chromatograms shown in Fig. 4 and compared with the behaviour of the related steroids unsubstituted at C-2. The reason why these compounds cannot be identified with certainty is that the characteristic differences in mobility of the four isomeric saturated 3-alcohols (which holds for the series based on androsterone, 11-oxoandrosterone, 11β-hydroxyandrosterone, tetrahydrocortisone and tetrahydrocortisol), and the still more characteristic changes in relative mobility on acetylation, are likely to be altered by the presence of the large methyl group on the adjacent C-2, particularly the latter changes which most probably depend on the 'effective polar group' of the acetoxyl group having the opposite conformation to that of the hydroxyl group of the original steroid. However, the identification of the 11-substituent in these steroids, with which we are mainly concerned, is as reliable as with the other steroids considered here.

The reliability of our methods of estimation depends largely on three sources of error. The first is the error in hydrolysis and extraction of the conjugated steroids in the urine, which is unknown for most of the 2α-methyl steroid metabolites. All the figures obtained have been corrected therefore by factors based upon experience with the natural steroid metabolites. These factors are largely determined by the partition coefficient of each steroid between the extracting solvent and water, which in turn parallels closely the polarity of each steroid as judged by its behaviour on chromatograms. Correction factors have therefore been applied to each 2α-methyl metabolite which match those found by experiment to correct for the extraction losses of a steroid having a similar mobility on these chromatograms. These factors are given in the tables to enable back-calculation of the actual amounts found. The assumption that glucuronosides of 2α-methyl steroids would be completely hydrolysed by our technique was confirmed by our failure to obtain any further 17-oxo steroids by applying Norymberski's technique of oxidative fission to aqueous residues which had been extracted previously by the standard technique using enzymic hydrolysis.

The second error is in the method of estimation by scanning chromatograms. The 95% confidence limits of this method have been approximately ±3% with blue tetrazolium and ±6% with alkaline *m*-dinitrobenzene over the last 3 years with the usual steroid metabolites, and there is no reason to suppose that results with the 2α-methyl steroids are less accurate. An additional error is introduced when this method is combined with bismuthate oxidation (Norymberski, 1952). This has been allowed for by using 2α-methylcortisol and 2α-methylcortisone as models for the whole process; a calibration curve is shown in Fig. 7.

The third error is in the extinction values used with the 17-oxo steroids (Wilson, 1954). Since it has been found that the main source of variation here is due to presence of 11-oxo or 11β-hydroxy groups which exert opposite effects, that other groups exert much smaller effects in the scanning method, and that the 2α-methyl group produced a slight fall in extinction, 2α-methyl-11β-hydroxyandrost-4-ene-3:11-dione and 2α-methyladrenosterone have been used as standards for the 2α-methyl metabolites, the former for the 11β-alcohols and the latter for the 11-ketones. It is unlikely therefore that this factor contributes an error of more than about ±5% to the corrected figure given in this paper; furthermore, such errors would be in one direction and would not affect the comparisons we wish to make.

In Expts. *B* and *C* two determinations of the naturally excreted steroid metabolites are given. These were carried out with 4 weeks' interval between them. As expected from earlier work, the second set of figures are all rather lower than the first, the relative proportions remaining very similar. Duplicate determinations carried out at the same time have agreed to within ±10% for each individual steroid during the last 3 years. As with most work of this type, the corrected figures given here have 95% confidence limits of approximately ±10% for the scanning methods and ±20% for the NaOH fluorescence method. This is sufficient for the argument of this paper.

The pattern of metabolites observed

The metabolites of cortisone and adrenosterone were entirely similar to those expected from earlier work (Dorfman, 1954; Burstein, Savard & Dorfman, 1953; Savard, Burstein, Rosenkrantz & Dorfman, 1953; Bradlow & Gallagher, 1958), although the ratio of 5α/5β metabolites of adrenosterone was lower than expected (2·1 instead of 4·0). While this may be due to the concomitant administration of prednisolone, the contrast between this steroid and cortisone (Dorfman, 1954) is still pronounced. Thus the ratio 11β-hydroxyandrosterone/(11β-hydroxyaetiocholanolone + 11-oxoaetiocholanolone) (after subtraction of the probable endogenous contribution) was 2·1 for adrenosterone (Expt. *D*) and 0·14 for (Expt. *A*).

*allo*Cortol was found as a metabolite of cortisone; this was expected from the finding of *allo*cortol as a metabolite of *allo*tetrahydrocortisone (Bush & Mahesh, 1959*a*). The ratio of 5α-H/5β-H steroids in

the 4:5-dihydro-3:20-diol group of hexahydro metabolites was of the same order as in the 4:5-dihydro group of tetrahydro metabolites (24 and 22·5% respectively). These and earlier results (Bush & Willoughby, 1957) give further support to the suggestion of Fukushima & Gallagher (1957) that 5α-H-3α-OH metabolites of cortisol will be of greater importance than was suspected previously.

The metabolites of the 2α-methyl steroids were remarkable for the large numbers and amounts of steroids retaining the Δ^4-3-oxo group compared with the very small amounts of saturated 3-hydroxy metabolites. This was expected, and the apparent inhibition of the reduction of the Δ^4-3-oxo group, which is the main reaction responsible for inactivation of the natural hormones, has previously been suggested as one reason for the enhanced biological activity of 2α-methylcortisol (Liddle *et al.* 1956). Despite this fact, it was remarkable that the greater part of all these metabolites, with the exception of 2α-methyladrenosterone and the related 11β-alcohol, were apparently conjugated, since very little could be extracted before enzymic hydrolysis of the urine, or before hydrolysis of the extract obtained by the resin method, which contains both free and conjugated steroids (Bush & Gale, 1957). This suggests that conjugation at positions 6, 20 or 21 must have occurred, which is interesting since the greater part of the 21-hydroxysteroid tetrahydrocortisone is excreted normally as the 3-monoglucuronoside (Schneider, Lewbart, Levitan & Lieberman, 1955) and similar evidence has been obtained for β-cortolone (J. J. Schneider, personal communication) and for tetrahydrocortisol and *allo*tetrahydrocortisol (I. E. Bush & M. Gale, unpublished work). It seems likely that conjugation at positions other than 3 in the natural steroid metabolites with a 3α-hydroxyl group is a slow reaction compared with the excretion of monoglucuronosides by the hepatic cell. This supposition is based upon the fact that it has been found that the yield of unchanged precursor excreted as monoglucuronoside is about twice as large when the precursor is a 3α-hydroxy steroid as with the related Δ^4-3-oxo steroids (e.g. compare Bush & Mahesh, 1959*a*, with the above; J. J. Schneider, personal communication). This suggests that most dehydrogenations and hydroxylations of the steroid molecule *in vivo* are reactions which are slow compared with the formation and excretion of 3-monoglucuronosides from hepatic cells. In the absence of a 3α-hydroxyl group, however, the slower formation of conjugates with 6-, 20- or 21-hydroxyl groups could occur to a considerable extent if the excretion of free steroid by hepatic cells were a slow reaction: this appears to be so, since more than 98% of the total 2α-methyl steroid metabolites were in conjugated form.

The other striking feature of the metabolism of the 2α-methyl steroids was the far greater percentage yield of 20-hydroxy metabolites of the C_{21} precursors, and of 6β-hydroxy metabolites of both the C_{21} and C_{19} precursors than is obtained with the related non-methylated steroids. This contrast seems also to be due, with the natural steroids, most probably to the predominance of the reactions: Δ^4-3-ketone → saturated 3α-alcohol → 3-monoglucuronoside → hepatic excretion. It seems likely that, as with the position of conjugation, the almost complete absence of these reactions with the 2α-methyl steroids allows the slower reactions of 20-hydrogenation and 6β-hydroxylation to proceed to a far greater extent. Although changes in enzyme–substrate affinity almost certainly play a part in these findings (Tomkins, 1956), the role of competing reactions suggested here seems of greater probable importance in view of the similar results in this respect obtained with steroids as different as 2α-methylcortisone, 2α-methylcortisol, prednisone, prednisolone and 2α-methyladrenosterone.

Liddle *et al.* (1956) studied the metabolic fate of 2α-methylcortisone and 2α-methylcortisol in terms of the rates of disappearance of Porter–Silber-reactive material from plasma, and the appearance of such material which was released from plasma by treatment with β-glucuronidase. They found only minor amounts of conjugated material after intravenous injection of these precursors, compared with the large amounts which appeared after administering hydrocortisone itself. This might appear at first to be in disagreement with our finding that most of the 2α-methyl steroid metabolites were in conjugated form. This, however, is not so, since the Porter–Silber reaction depends upon the presence of the 20-oxo group of the dihydroxyacetone side chain, and one of the main features of the 2α-methyl steroids is that the great majority of their metabolites have been reduced at position 20 to the related 20ξ-alcohols. Such metabolites would not have been measured by Liddle *et al.* (1956) with their methods. These authors also found that the ring-A reducing system of rat liver (Tomkins, 1956) would reduce 2α-methylcortisone but not 2α-methylcortisol. This would agree with the total lack of reduction in ring A that we observed with 2α-methylcortisol, but the figures given in the table of Liddle *et al.* would not explain the almost equally inefficient reduction of ring A of 2α-methylcortisone in our experiments, since they found no difference between cortisone and 2α-methylcortisone with this enzyme system. However, qualitative agreement between human metabolism *in vivo* and that of the rat *in vitro* is indicated, since only with 2α-methylcortisone were we able to find a saturated 3-hydroxy metabolite (ME3; XIX, Chart 3),

although this made up only 21% of the total recognized metabolites, or 4% of the ingested precursor. Such metabolites made up 99% or more of the total recognized metabolites of cortisone, or 45% of the ingested steroid.

The pattern of 11-*oxo and* 11β-*hydroxy metabolites*

It is clear from the results that the pattern of metabolites of cortisone and adrenosterone are those expected from previous work. Thus the 5α-H saturated 3α-OH metabolites are largely or entirely in the 11β-hydroxy form, as are the Δ^4-3-ketones themselves. On the other hand, their 5β-H saturated metabolites are predominantly in the 11-oxo form, the ratio 11-ketone/11β-alcohol being approximately 2/1 in each pair. In contrast, the metabolites of 2α-methylcortisone and 2α-methylcortisol are largely in one or other form according to the nature of the 11-oxygen function in the precursor. Thus with 2α-methylcortisone the ratio total 11-ketones/total 11β-alcohols was 15·8/1, while with 2α-methylcortisol this ratio was 0·16/1. Similarly, the plasma steroids were in similar ratios after oral administration, 2α-methylcortisone contrasting strongly with cortisone in this respect; the latter, as shown here and previously (Bush, 1956), was almost entirely reduced to cortisol in the plasma after oral administration, the small amount of material which might have been unchanged cortisone being too small to be identified with certainty.

With 2α-methyladrenosterone the same general effect was noticed, but to a smaller degree than with 2α-methylcortisone. Thus the ratio total 11-ketones/total 11β-alcohols was 1·8/1 in the urinary metabolites. The contrast with adrenosterone itself was well shown in the plasma, where the ratio adrenosterone/11β-hydroxyandrost-4-ene-3:17-dione was 1/9 after oral adrenosterone, while the comparable ratio for the 2α-methyl analogues after 2α-methyladrenosterone was 1/1. Reduction of the 11-oxo group in 2-methyladrenosterone is therefore clearly less complete than in adrenosterone itself, but the inhibition of the reduction is not so pronounced as in the cortisone analogue.

Reduction of 11-*oxo groups*

In the previous paper (Bush & Mahesh, 1959*a*) we showed that reduction of steroid 11-oxo groups occurred readily with steroids of the 5α-H-3α-OH but not with those of the 5β-H-3α-OH type. This is readily understood on stereochemical grounds, since the buckled A/B ring system of the 5β-H steroid would be expected to hinder the approach of the 11-oxo group to the active site of an enzyme which associates with the steroid by the α-surface of the latter. This is the most likely mode of association, since reduction to the 11β-hydroxy form requires that the α-bond of the carbonyl group be attacked by hydrogen, and this is most likely to be in the form of reduced coenzyme attached to the enzyme surface (Vennesland & Westheimer, 1954). Whether this reaction is carried out by one enzyme or several, it seems that the active region of such enzymes is similar in requiring a flat α-surface to the steroid substrate for efficient attack upon the α-carbonyl bond.

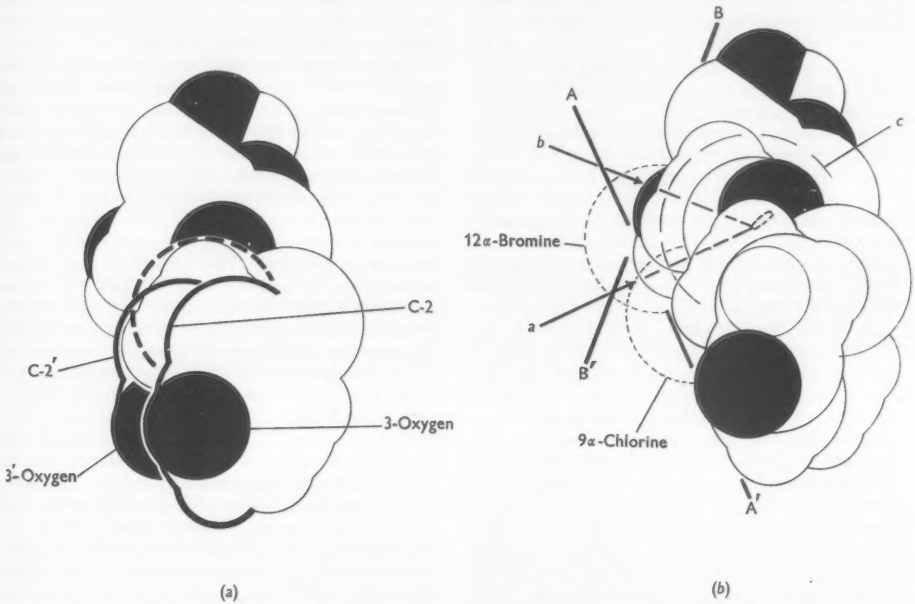
While this simple conception suffices to explain the results with non-methylated steroids, it does not explain the behaviour of 2α-methyl steroids, nor does it entirely explain the behaviour of the 9α-chloro steroids. In Fig. 8 two sets of projections are shown for typical steroids examined in these studies. These have been constructed by using the positions of carbon atoms in rings B, C and D derived from X-ray studies (Crowfoot, 1944; Shoppee, 1946), and the conformations of ring A derived from X-ray studies for 5α-(H) and 5β-(H) steroids, and from models for the unsaturated A rings. The van der Waals radii are taken directly from Pauling (1940), since the larger values given by him are more appropriate for this type of problem, which involves molecular associations similar to those of the solid state, rather than the smaller values, which are better for explaining intramolecular steric hindrance (Braude & Sondheimer, 1955). The former situation is largely governed by repulsions in the line of the bonds; the latter largely by repulsions normal to the bonds involved, and complicated by the consideration of excited states and larger energy barriers. It is easily seen that the 2α-methyl group of steroid Δ^4-3-ketones, which is equatorial, projects only a small distance beyond the plane of the α-surface of rings C and D. It projects, in fact, 1·4 Å less than C-2 and C-3, and 1·6 Å less than O-3, of prednisone, which is known to be reduced to prednisolone, although less completely than cortisone is reduced to cortisol (Bush & Mahesh, 1958*b*). Again, the reduction of 2α-methyladrenosterone is more complete (35% of all metabolites) than that of 2α-methylcortisone (6% of all metabolites), suggesting that the 17β-side chain of the latter is involved in hindering association with the active region of the enzyme. These findings could be explained by assuming that the active site of the steroid 11β-dehydrogenase resembled a shallow pit with a sharp quarry-like edge closely apposed to the outer edge of the steroid at C-2, C-16, C-17, C-20 and C-21. The absence of the side chain at C-17 would then allow 2α-methyladrenosterone to associate more closely with the active region by rotation about the 11-oxo group on an axis normal to the main plane of ring C, anti-clockwise as the formula is usually drawn, to let the 2α-methyl group slip down 'over the edge' at C-2. However,

this explanation will not allow for the apparently quite good apposition of 9α-chlorocortisone (I. E. Bush & V. B. Mahesh, unpublished work). The only explanation that covers all the findings satisfactorily at present is that hydrogen attack on the α-carbonyl bond with this enzyme occurs from the 'north-west' direction in the projection (Fig. 8) rather than from 'due west' and that the steroid associates with the active region of the enzyme with the two surfaces at an angle (projection *b*, BB′) rather than with the face-to-face apposition assumed in the original simple theory (projection *b*, AA′; Fig. 8).

These speculations may seem too bold for results obtained from experiments *in vivo*, but we have already given reasons for supposing that they are justifiable and that the many other factors that might be involved in producing our results largely cancel one another when they are evaluated for the wide variety of precursors that have been studied (Bush & Mahesh, 1958*b*). However, they require confirmation by experiments with other steroid precursors and by experiments *in vitro*.

Correlation of 11*-oxygen functions with biological activity*

In a previous paper (Bush, 1956) it was suggested that the biological effects of the 11-oxygen function of adrenocortical steroids could not be explained on the basis of simple solubility effects or of an activation of some other part of the steroid molecule. One had instead to suppose that this oxygen function was itself involved in a highly specific interaction with the 'receptor site' for such



(*a*) (*b*)

Fig. 8. (*a*) Composite projection on a plane normal to a line joining C-8 and C-10 of cortisone, prednisone and 2α-methylcortisone. C-2, C-2 of cortisone and 2α-methylcortisone; C-2′, C-2 of prednisone; 3-oxygen, as in cortisone and 2α-methylcortisone; 3′-oxygen, as in prednisone. The heavy line is the α-side profile of C-2 and C-3 and of the 4-H atom. The heavy dashed line is the profile of the 2α-methyl group. Because of conjugation the repulsion between the 2α-methyl and 3-oxo groups has been assumed to lead only to bending and slight lengthening of the 2α-methyl–C-2 bond. This repulsion was calculated with 'small' van der Waals radii: all other van der Waals radii are taken from Pauling (1940) (see text). The conformation of the side chain is not necessarily the preferred one. (*b*) Hypothetical projection of possible association between cortisone and active region of the 11β-dehydrogenase. AA′, plane of enzyme 'surface' of simplified theory (Bush & Mahesh, 1958*b*) with direction of hydrogen attack on α-carbonyl bond, *a*; BB′, more probable orientation of enzyme 'surface' and hydrogen attack, *b*, to account for relative hindrance of 2α-methyl group, the A ring of prednisone, the influence of the 17β-side chain and of the 9α-chloro and 12α-bromo groups (see text); *c* is the circumference within which a hydrogen atom or hydride ion must approach C-11 before hydrogenation is more likely than recoil.

hormones. In the light of evidence available at that time, two possibilities were raised: either the known oxidation–reduction reaction of the 11-oxygen function was the basis of the biological actions of such hormones, being linked with some essential redox system; or the 11β-hydroxy forms were active at the receptor site but not the related 11-ketones, which were active after systemic administration only because of their rapid reduction to the former. The results given above clearly suggest that the latter hypothesis is correct, thus placing considerable limitations on the type of chemical process underlying the biological actions of these hormones.

The original biological findings, with the exceptionally large difference between the biological activities of 2α-methylcortisone and 2α-methylcortisol, strongly supported this hypothesis (Liddle *et al.* 1956; Peterson *et al.* 1957) but were not a satisfactory proof by themselves. Since we have been able to show that all the metabolites of 2α-methylcortisone are largely unreduced at C-11, and that the absorption and distribution of 2α-methylcortisone is not greatly different from that of 2α-methylcortisol, it seems clear that this exceptionally low activity of the 11-ketone is not easily accounted for except by supposing that it is itself inactive at the receptor site. The small activity actually possessed by 2α-methylcortisone is reasonably attributed to the very small amount of 2α-methylcortisol to which it gives rise in the plasma.

Such results not only favour the hypothesis that biological activity is confined to the 11β-hydroxy forms of the 11-oxygenated adrenal steroids, but seem to exclude the alternative possibility that the reversible 11β-dehydrogenase reaction is itself the basis of their biological activity. Since Talalay & Williams-Ashman (1958) have recently tentatively favoured this very alternative, on the basis of a penetrating analysis of Villee's (1955) findings with oestradiol and placental *iso*citric dehydrogenase, it is essential to consider just how far our evidence, however suggestive, can be accepted as exclusive of such hypotheses. The most powerful argument against our interpretation at first sight is the usual objection to metabolic studies of this type being used to infer connexions between overall metabolism and pharmacological effects; since only 19·33 % of the dose of hormone has been accounted for as metabolites, one does not know what reactions the remainder has undergone. In this sense our interpretation cannot be considered exclusive. However, it is reasonably certain that the active hormone in the tissues is rapidly exchanged with the circulating hormone, and the steady-state ratio 11-ketones/11β-alcohols in plasma must be a definite function of the same ratio in the tissues. If the basic mechanism of action of this type of hormone depended on the 11β-dehydrogenase reaction, then the activity would depend on the turnover rate of the reaction which, for a hormone such as 2α-methylcortisol which is more active than cortisol (Dulin *et al.* 1957), would have to be at least comparable with that of cortisol. In this case the steady-state ratio 11-ketone/11β-alcohol in tissues and in plasma, with the known time factors involved (Peterson *et al.* 1957; Ayres *et al.* 1957), would be the same whether the steady state were approached from the direction of the ketone or the alcohol. In actual fact, 4·75 hr. after administering 2α-methylcortisol, this ratio was 1/37·5, and 3·33 hr. after administering 2α-methylcortisone it was 10/1. Unless one invokes the theory that hitherto undiscovered modifications of such hormones are actually active at receptor sites, these results are incompatible with the theory that these two steroids were undergoing rapid interconversion of the type required by the hypothesis of Talalay & Williams-Ashman (1958).

Various other attempts can be made to reinterpret our results to fit the redox theory of biological action, but they will not be explored here since they all involve unproven or unlikely complications which make them unacceptable on Occam's principle. Two other examples reinforce the argument given here. First, we have found that prednisone (15 mg./24 hr.) is relatively inefficient in suppressing adrenal secretion in man, whereas prednisolone (10 mg./24 hr.) is as efficient as cortisone (37·5 mg./24 hr.). Hodges & Vernikos (1958) have found the same thing with rats. This is correlated with a partial inhibition of the reduction of prednisone such that the plasma concentration ratio prednisone/prednisolone is approximately 1/1. (Bush & Mahesh, 1958*b*). Secondly, Herz, Fried & Sabo (1956) have found that 12α-bromo-11β-hydroxyprogesterone has about one-third the activity of cortisone in the liver-glycogen deposition test, whereas the related 11-ketone is inactive. These compounds are at present under study, but it can be seen from the projection *b* (Fig. 8) that the large bromine atom, with an axial conformation, presents the largest steric hindrance to association with the 11β-dehydrogenase of all the steroids so far considered in these studies. Again, the inactivity of the 11-ketone is probably due to its failure to undergo reduction to the 11β-alcohol *in vivo*.

Thus three exceptional pairs of steroids are known in which the 11-ketonic forms are less active, compared with the 11β-hydroxy forms, than is found with the natural adrenal steroids, although the 11β-hydroxy forms are more active than their parent hormones.

In two of these cases a partial or almost complete inability to undergo reduction of the 11-oxo

group is associated with a partial or almost complete loss of biological activity; in the third the inactivity of the ketone is associated with the possession of a group almost certain to prevent this reduction from occurring. One is left with the almost inescapable conclusion that the biological activity of 11-oxygenated steroids as glucocorticoids is due to the specific interaction of an 11β-hydroxyl group with the receptor sites for such hormones, and that this interaction does not involve oxidation–reduction at C-11. While it is possible that this interaction is with an enzyme carrying out a redox reaction at C-20 (no other group capable of such reactions is common to all known glucocorticoids), this does not seem likely at present.

Although these considerations leave much to be done before the mode of action of the glucocorticoids is understood on a molecular level, they provide reasonable grounds for excluding a large number of mechanisms that have been postulated previously. Thus, in agreement with Hechter (1955), it seems less and less profitable to pursue the 'coenzyme' theory of hormone action, at any rate for redox reactions. Rather, one must consider the physical actions of such hormones that might result from their specific interaction with receptor sites. This is being investigated.

SUMMARY

1. The metabolism of 2α-methylcortisone, 2α-methylcortisol and 2α-methyladrenosterone was compared with that of cortisone and adrenosterone after oral administration to young men, with special reference to oxidation–reduction of the 11-oxygen function of these steroids.

2. The 2α-methyl steroids showed four main features of interest: There was an almost complete lack of metabolites reduced in ring A; there was a much higher proportion of 6β-hydroxy and 20ξ-hydroxy metabolites; there was an inhibition of the usual interconversion of 11-ketones and 11β-alcohols, which was far more complete with 2α-methylcortisone than with 2α-methyladrenosterone and, despite the lack of 3α-hydroxy metabolites, at least 98 % of the recognized metabolites were in conjugated form.

3. The extent of reduction of the 11-oxo groups of the precursors suggested by the pattern of urinary metabolites was paralleled in each case by the 11-ketone/11β-alcohol ratio in plasma collected up to 3 hr. after taking each precursor. Furthermore, there was little oxidation of the 11β-hydroxyl group of 2α-methylcortisol.

4. Arguments are given suggesting that association of 11-oxo steroids with the 11β-dehydrogenase occurs with the steroid tilted on the active region of the enzyme, so that contact is mainly over the α-surface of the molecule in the region of carbon atoms 1, 2, 11, 12, 17 and 21.

5. The exceptionally high ratio of biological activities of 2α-methylcortisol and 2α-methylcortisone appears to be correlated with the exceptionally poor reduction of the latter to the former. It is concluded that biological activity of the 11-oxygenated adrenal steroids is confined to the 11β-hydroxy forms, except when the 11-ketones can be reduced to the former, and that the oxidation–reduction of the 11-oxygen function occurring with the natural adrenal hormones of this type is not directly involved in their mode of action. Alternative theories are discussed.

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Studies on the Biosynthesis of Riboflavin

5. GENERAL FACTORS CONTROLLING FLAVINOGENESIS IN THE YEAST *CANDIDA FLARERI**

BY T. W. GOODWIN AND D. McEVOY
Department of Biochemistry, The University, Liverpool 3

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Among the flavinogenic fungi most studied from the point of view of the biosynthesis of riboflavin are the Ascomycetes *Eremothecium ashbyii* (Schopfer, 1944; MacLaren, 1952; Goodwin & Pendlington, 1954; McNutt, 1954, 1956; Brown, Goodwin & Pendlington, 1955; Goodwin & Jones, 1956; Brown, Goodwin & Jones, 1958) and *Ashbya gossypii* (Tanner, Vojnovich & van Lanen, 1949; Plaut, 1954*a*, *b*; Plaut & Broberg, 1956). Certain yeasts of the genus *Candida*, e.g. *C. guilliermondia* (Burkholder, 1943) and *C. flareri* (Tanner, Vojnovich & van Lanen, 1945), are also good riboflavin producers, but have not been examined in such detail. The present investigation was undertaken to define more precisely the general factors controlling flavinogenesis in *C. flareri* and to compare the results with those already obtained in this Laboratory with *E. ashbyii*. *C. flareri* was preferred to *C. guilliermondia* because it synthesizes considerably more riboflavin (Tanner *et al.* 1945).

The most critical constituent of the medium for flavinogenesis in *Candida* spp. is undoubtedly iron; the optimum concentration is 0·005–0·01 μg./ml. and higher concentrations are strongly inhibitory (Tanner *et al.* 1945; Levine, Oyaas, Wasserman, Hoogerheide & Stern, 1949). This permissible concentration of iron can be increased ten times by controlling the amount of cobalt in the medium (10 μg./ml.) (Enari, 1955). According to Levine *et al.* (1949), other trace elements (manganese, copper, zinc, tin, nickel and aluminium) up to 0·10 μg./ml. have no inhibitory effect on flavinogenesis, whereas zinc at slightly higher concentrations (0·2–0·8 μg./ml.) is stimulatory (Schopfer & Knüsel, 1956).

Because the significance of iron was not known when the original investigations on *Candida* spp. were carried out (Burkholder, 1943, 1944) the results obtained must be considered quantitatively suspect.

Both *C. guilliermondia* and *C. flareri* will produce riboflavin on a medium containing ammonium sulphate or urea as the sole nitrogen source, but urea is rather more effective in *C. flareri* (Levine *et al.* 1949). Supplementation of a medium containing ammonium sulphate as its sole nitrogen source with asparagine, glycine or urea stimulated further flavin production in *C. guilliermondia* and *C. flareri* (Burkholder, 1944; Levine *et al.* 1949).

Aeration or agitation or both is required for good yields of riboflavin, which reach its peak 3–5 days after inoculation (Levine *et al.* 1949).

Some of the present results have already been briefly reported (Goodwin & McEvoy, 1957).

* Part 4. Brown, Goodwin & Jones, 1958.

EXPERIMENTAL

Organism. The organism used throughout was *Candida flareri*, NRRL 245, kindly provided by the North Regional Research Laboratories, Peoria, Ill., U.S.A. It was maintained by fortnightly transfers on Sabouraud medium–agar slopes.

Media. The basal medium contained (in 100 ml.): glucose, 2·0 g.; $(NH_4)_2SO_4$, 0·873 g.; KH_2PO_4, 0·05 g.; $MgSO_4,7H_2O$, 0·05 g.; $CaCl_2$, 0·03 g.; biotin, 0·1 μg.; B (as $Na_2B_4O_7,10H_2O$), 1 μg.; Mn (as $MnSO_4,4H_2O$), 1 μg.; Zn (as $ZnSO_4,7H_2O$), 7 μg.; Cu (as $CuSO_4,5H_2O$), 1 μg.; Mo [as $(NH_4)_6Mo_7O_{24},4H_2O$], 1 μg.; Fe as [$FeSO_4,(NH_4)_2SO_4,6H_2O$], 1 μg. This medium is similar to that of Burkholder (1943, 1944) except that the concentrations of $(NH_4)_2SO_4$ and iron are lower. The trace elements and $(NH_4)_2SO_4$ were stored in concentrated solutions and added in appropriate amounts to the media just before autoclaving. All the chemicals were of analytical grade; the deionized water used was that obtained from an Elgastat and had an electrical conductivity of not less than 800 000 Ω. To eliminate sources of error from contamination with traces of iron, all glassware was soaked in chromic acid for 24 hr., washed with tap water and then with deionized water, and dried in an oven at 150°. The further precaution of autoclaving the glassware completely filled with deionized water at 20 lb./in.^{2} for 30 min. before drying (Waring & Werkman, 1942) was found in preliminary experiments not to be necessary. The pH was adjusted to 5·0 and the medium was sterilized by autoclaving at 15 lb./in.^{2} for 15 min. Experimental media made by replacing $(NH_4)_2SO_4$ by amino acids and purines (obtained from L. Light and Co., Colnbrook, Bucks., British Drug Houses Ltd., Poole, Dorset, or Roche Products Ltd., Welwyn Garden City, Herts) in the basal medium were sterilized in the same way.

Cultural conditions. Media (15 ml.) were dispensed in 75 ml. Erlenmeyer flasks, inoculated with three drops of a spore suspension drawn from a 2 days' culture grown on the basal medium and the flasks incubated in a rotary shaker in a room maintained at 28$\mp$1°. [We are very grateful to Roche Products Ltd. (Dr A. L. Morrison) for the loan of the prototype of the shaker used in the present investigation.] It had been previously reported that shaking is necessary for good yields of riboflavin (Burkholder, 1944); we confirmed this and noted that static cultures of *C. flareri* produced almost no riboflavin. The use of a 2 days' inoculum was prompted by the fact that it is most suitable with *A. gossypii* (Tanner *et al.* 1949) and *E. ashbyii* (Goodwin & Pendlington, 1954) and that it is recommended in much patent literature (see Goodwin, 1959). It is essential to maintain standardized inoculating conditions, for Burkholder (1944) found that increasing the size of the inoculum decreased riboflavin production.

Removal of iron. When necessary, excess of iron was removed from media by shaking with a solution of 8-hydroxyquinoline in chloroform (Waring & Werkman, 1942; Tanner *et al.* 1945).

Analytical procedures. In general four replicate flasks were examined in each experiment, and the dry weight of the cells and the riboflavin levels in the media were determined as previously described for *E. ashbyii* (Goodwin & Pendlington, 1954). In *Candida* spp. essentially all the riboflavin is present in the culture medium; no significant amount is retained in the mycelium (Burkholder, 1944). In this the organisms differ from *E. ashbyii* and *A. gossypii*, which can retain considerable amounts in the mycelium (Goodwin, 1959).

The methods of Brown *et al.* (1958) were used for chromatographic separation and spectrophotometric determination of purines.

RESULTS

Reproducibility of results

In an experiment designed to assess the reproducibility of results in replicate flasks, 20 shake cultures grown for 3 days at 28° on 15 ml. of basal medium in 75 ml. conical flasks were examined. The results were as follows: dry wt. (mg.), mean 54·6 $\mp$ S.D. 0·83, range 52·6–55·8; riboflavin (μg./15 ml.), mean 206 $\mp$ S.D. 11·6, range, 174–224; riboflavin (mg./100 g. of dry mycelium), mean 377 $\mp$ S.D. 22·9, range 318–413. Because of these results it was considered necessary generally to carry out subsequent analyses only in quadruplicate.

Effect of the incubation period on growth and flavinogenesis

In the basal medium under our conditions, growth and flavinogenesis are essentially complete after incubation for 2–3 days (Fig. 1), and after a

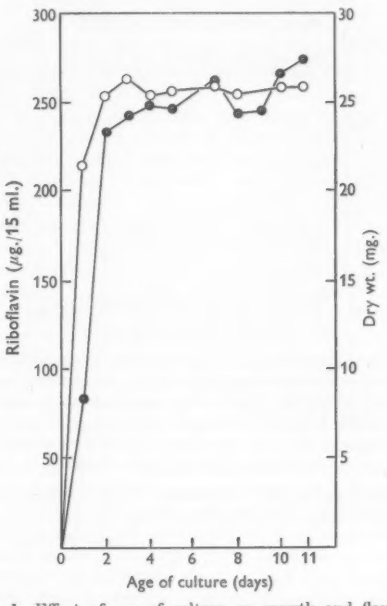

Fig. 1. Effect of age of culture on growth and flavinogenesis in *C. flareri*. Amounts were produced in 15 ml. of basal medium in 75 ml. flasks; shake culture; temp. 28°. ●, Riboflavin; ○, dry wt.

further 8 days there is little significant change. Because of these results all later analyses were carried out on 3 days' cultures.

Effect of iron on flavinogenesis

Addition of iron to the basal medium (Fig. 2) shows little significant effect below 2·0 μg./100 ml., but above this concentration there is a very marked decrease in flavinogenesis. There is no effect on growth of the organism. These results indicate that the iron content of the basal medium was of the right order to support maximum flavinogenesis. Because of this confirmation of previous workers' observations on the effect of higher concentrations of iron on flavinogenesis by *Candida* spp., in experiments involving additions of extra constituents, the effect of shaking with 8-hydroxyquinoline was generally examined. This procedure was especially important if the substance under test was considered to be a stimulator of flavinogenesis and was found on first investigation not to be so.

Leviton (1946) found that if the iron content were not too high the sensitivity of the flavinogenic system in the anaerobic *Clostridium acetobutylicum* to iron could be overcome by the addition of catalase. The explanation given was that H_2O_2 produced metabolically would in the presence of iron rapidly oxidize riboflavin. Addition of catalase destroyed the H_2O_2 and thus protected the riboflavin. This approach was examined with *Candida flareri*; a known amount of iron was added to the basal medium so as to make the final concentration 0·1 p.p.m., and after incubation for 24 hr. a solution of catalase (L. Light and Co. Ltd.) was added to one set of flasks. The results (Table 1) show that with this catalase does not overcome the inhibitory effect of excess of iron. Furthermore, if riboflavin is added to a culture of *C. flareri* growing on an iron-rich medium there is no destruction of this added riboflavin. In *Clostridium acetobutylicum* added riboflavin is destroyed in a medium rich in iron. It is obvious that excess production of H_2O_2 resulting from a deficiency of catalase is not the explanation of the sensitivity to iron of the flavinogenic system in *C. flareri*. The same conclusion was recently reached with the closely related *C. guilliermondia* (Shavlovskii & Chistiakova, 1956).

Effect of other trace elements

The removal *en bloc* of the other trace elements (B, Mn, Zn, Cu and Mo) recommended by Burkholder (1943) reduced markedly riboflavin production, whereas growth was only slightly affected (Table 2). In this experiment L-asparagine was

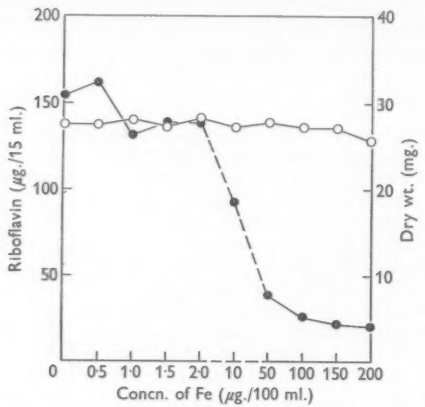

Fig. 2. Effect of iron on growth and flavinogenesis in *C. flareri*. Basal medium + varying amounts of Fe as $FeSO_4(NH_4)_2SO_4,6H_2O$; other conditions were as given in Table 1. ●, Riboflavin; ○, dry wt.

Table 1. *Effect of added catalase on riboflavin production by* Candida flareri *on an iron-rich medium*

Amounts produced in 75 ml. conical flasks containing 15 ml. of basal medium plus 0·1 μg. of iron/ml., with and without the addition of catalase (5 Kiel* units/flask) (added 24 hr. after inoculation); 3 days' shake cultures; temp. 28°.

Concn. of Fe in medium (μg./ml.)	Riboflavin (μg./15 ml.)	
	Catalase absent	Catalase present
0·01	294	410
0·1	146	171

* A Kiel unit is the quantity of catalase necessary to decompose 1 g. of 100% H_2O_2 in 10 min. at 25° in an inert atmosphere of CO_2 or N_2.

Table 2. *Effect of trace elements on growth and riboflavin synthesis by* Candida flareri

Basal medium plus L-asparagine (0·2 g./100 ml.) with and without addition of trace elements B, Mo, Zn, Cu and Mn. 2, 4 and 7 days' shake cultures; temp. 28°: 15 ml. of medium in 75 ml. conical flasks; triplicate determinations.

	2 days' cultures		4 days' cultures		7 days' cultures	
	Dry wt. (mg.)	Riboflavin (μg./15 ml.)	Dry wt. (mg.)	Riboflavin (μg./15 ml.)	Dry wt. (mg.)	Riboflavin (μg./15 ml.)
Basal medium with trace elements	61·1	520	53·9	610	52·3	502
Basal medium without trace elements	49·9	393	49·9	313	42·4	212

added to our basal medium in order to reproduce Burkholder's conditions exactly. In fact L-asparagine is not necessary for flavinogenesis (see Table 6). This problem was not pursued further, but the result indicated that at least one other trace element is concerned in flavinogenesis; in this connexion Schopfer & Knüsel (1956) indicated that zinc was important.

Effect of glucose and phosphate

These two compounds are grouped together because their reported effect on flavinogenesis is similar. According to Burkholder (1943) increasing the concentration of phosphate or of glucose in the medium inhibited flavinogenesis; we have confirmed both these observations and have further shown that the effects are due to the extra iron added to the medium in increasing the concentrations of phosphate and glucose. If the solutions of these constituents are shaken with 8-hydroxyquinoline before being added to the basal medium, then the inhibitory effect vanishes (Table 3). It is obvious that, with our glucose, the concentration recommended by Burkholder (1944) and used throughout the present work produces slightly less than maximal amounts of riboflavin, and that the concentration (4 %) recommended by Tanner *et al.* (1945) is too high.

Table 3. *Effect of removal of iron on the inhibitory action on flavinogenesis by* Candida flareri *of high concentrations of glucose and phosphate*

Medium *A*: basal medium with glucose concn. increased to 4·0 % (w/v). Medium *B*: basal medium with KH_2PO_4 concn. increased to 0·0875 % (20 mg. of P/100 ml.). Iron was removed by shaking with 8-hydroxyquinoline. Other conditions were as described in Table 1 except that nine flasks were examined in each group.

	Riboflavin production (μg./15 ml.)	
Medium	Without removal of Fe	After removal of Fe
A	268	320
B	267	367

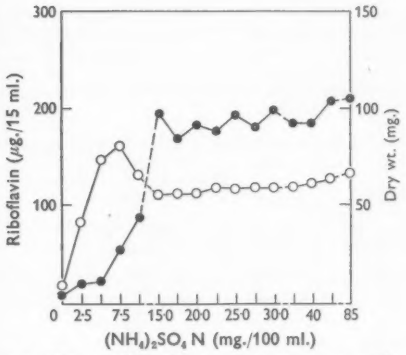

Fig. 3. Effect of $(NH_4)_2SO_4$ concentration on growth and flavinogenesis in *E. ashbyii*. Amounts in 15 ml. of basal medium with varying concentrations of $(NH_4)_2SO_4$; shake cultures; temp. 28°. Quadruplicate determinations. ●, Riboflavin; ○, dry wt.

Effect of varying the concentration of ammonium sulphate

Growth of *C. flareri* reaches its maximum with 7·5 mg. of $(NH_4)_2SO_4$ nitrogen/100 ml.; increasing the amount above 7·5 mg. of nitrogen/100 ml. results in decreased growth until it levels off at 15 mg. of nitrogen/100 ml. and above (Fig. 3). This type of growth curve is completely reproducible. Riboflavin production, on the other hand, increases rapidly to a maximum at 15 mg. of $(NH_4)_2SO_4$ nitrogen/100 ml. It may be significant that the greatest increase in flavinogenesis occurs over that range where a decrease in growth is noted.

It was found that NH_4NO_3 and ammonium acetate would satisfactorily replace $(NH_4)_2SO_4$ but that KNO_3 would not support growth of *C. flareri*.

Effect of certain amino acids

When $(NH_4)_2SO_4$ in the basal medium was replaced by various amino acids it was found that they could be divided into two groups: (*a*) those which stimulated growth very considerably and (*b*) those which supported equal or less growth (Table 4). Urea, which was also examined, was in the first group. The experiments were carried out at different times, and the growth of the $(NH_4)_2SO_4$ controls varied slightly from experiment to experiment; in order to collect the results into one table, growth is expressed as the percentage of that of the appropriate $(NH_4)_2SO_4$ control. In none of these experiments did the riboflavin levels reach those achieved on $(NH_4)_2SO_4$ alone. This could have been due either to the presence of iron in the amino acids added or to the fact that as growth was so much better in many cases than on $(NH_4)_2SO_4$, no residual nitrogen was available for channelling into riboflavin. All the strongly growth-stimulating amino acids and urea were therefore examined at higher concentrations, and Table 5 shows that under these circumstances (40 mg. of nitrogen/100 ml.) L-arginine and urea produce much more riboflavin than does $(NH_4)_2SO_4$, but as growth is also considerably increased the yield per unit dry weight of cells is of the same order in all three cases. Glycine, L-asparagine and $(NH_4)_2SO_4$ are equally flavinogenic, but as growth is much greater with the amino acids the yield per unit dry weight of

cells is very much less than on $(NH_4)_2SO_4$. None of the other amino acids examined, DL-serine, L-aspartic acid, DL-glutamic acid and DL-threonine, which stimulate flavinogenesis in *E. ashbyii* (Goodwin & Pendlington, 1954), and DL-ornithine and DL-citrulline, studied because of the activity of urea and L-arginine, supported flavinogenesis comparably with that achieved on $(NH_4)_2SO_4$. The possibility of the presence of iron in these amino acids had to be considered. After shaking the individual amino acid solutions with 8-hydroxyquinoline before their incorporation into the medium, it was found that flavinogenesis was considerably improved with serine and ornithine, very slightly stimulated with L-asparagine, unaltered with DL-threonine and DL-glutamate and, unexpectedly, reduced with DL-citrulline (Table 6).

Table 4. *Effect of different amino acids on the growth of* Candida flareri

Basal medium with $(NH_4)_2SO_4$ replaced by either 7·5 or 20·0 mg. of amino acid N/100 ml. DL-Amino acids were added at twice the normal concentration. Other conditions were as described in Table 1.

	Growth [% of that on $(NH_4)_2SO_4$]	
Nitrogen source	7·5 mg. of N/100 ml.	20 mg. of N/100 ml.
$(NH_4)_2SO_4$	100	100
DL-Serine	194	456
Glycine	179	489
L-Aspartic acid	199	568
L-Asparagine	164	488
L-Arginine	152	314
DL-Glutamate	209	535
L-Proline	179	500
DL-Citrulline	152	430
Urea	154	340
DL-Tryptophan	169	156
L-Alanine	195	119
DL-Ornithine	119	256
L-Valine	114	124
L-Tyrosine	33	150
DL-Threonine	29	49
L-Histidine	38	64
DL-Methionine	42	79
L-Lysine	86	86
L-Leucine	65	87
L-Cysteine	16	72
L-Phenylalanine	37	59
L-Hydroxyproline	7·3	11

Effect of purines and pyrimidines

As sole sources of nitrogen, xanthine and uric acid support good growth but no flavinogenesis, whereas adenine, adenosine and hypoxanthine support no significant growth (Table 7). When added at a level of 12·5 mg. of nitrogen/100 ml. to media also containing 7·5 mg. of $(NH_4)_2SO_4$ nitrogen/100 ml., then all purines stimulate growth considerably compared with a medium containing 20 mg. of $(NH_4)_2SO_4$ nitrogen/100 ml. Xanthine also stimulated flavinogenesis and uric acid was equal to $(NH_4)_2SO_4$. Adenine and hypoxanthine, on the other hand, showed no effect whatever on flavinogenesis. At its maximum level of solubility (5 mg./100 ml.), and in the absence of $(NH_4)_2SO_4$, guanine supported growth but did not stimulate flavinogenesis (Table 8). In the presence of $(NH_4)_2SO_4$ it stimulated both growth and flavinogenesis. The failure of adenine and hypoxanthine to stimulate flavinogenesis might have been due to contamination with iron; however, after shaking with 8-hydroxyquinoline before incorporation into the culture media it was found that the effect of hypoxanthine was unchanged. For a reason which at the moment remains inexplicable growth did not occur on the adenine medium shaken with 8-hydroxyquinoline.

By the methods of Brown *et al.* (1958) it was shown with adenine that in the presence of $(NH_4)_2SO_4$ and under the experimental conditions

Table 5. *Effect of high concentrations of certain amino acids on growth and flavinogenesis in* Candida flareri

Basal medium with $(NH_4)_2SO_4$ replaced by urea or an amino acid at N concentrations of either 20 or 40 mg./100 ml. Other conditions were as described in Table 1.

	Growth [% of that on $(NH_4)_2SO_4$]		Riboflavin [% of that on $(NH_4)_2SO_4$]	
Nitrogen source	20 mg. of N/100 ml.	40 mg. of N/100 ml.	20 mg. of N/100 ml.	40 mg. of N/100 ml.
$(NH_4)_2SO_4$	100	100	100	100
Glycine	520	576	18	125
DL-Serine	371	471	13	65
L-Asparagine	418	458	47	93
L-Aspartic acid	507	541	10	18
DL-Glutamate	513	549	17	23
DL-Ornithine	275	362	23	19
DL-Citrulline	359	435	14	18
L-Arginine	268	266	71	297
Urea	405	415	90	326
DL-Threonine	98	106	8	10

described in Table 8, about 40% of the added adenine is utilized and that no detectable amounts of extracellular hypoxanthine are produced; this differs from the observation with *E. ashbyii*, where considerable amounts of adenine are converted into hypoxanthine (Brown *et al.* 1958).

The pyrimidines thymine, uracil, cytosine and orotic acid, when used as sole sources of nitrogen did not support growth of *C. flareri*, and when incorporated into media containing $(NH_4)_2SO_4$ had no effect on either growth or flavinogenesis. These completely negative results are not recorded here.

Table 6. *Effect of removal of iron on the flavinogenic action in* Candida flareri *of high concentrations of certain amino acids*

Basal medium with different amino acids or $(NH_4)_2SO_4$ added at concentrations of 40 mg. of N/100 ml.; iron was removed from amino acid and $(NH_4)_2SO_4$ solutions before addition to basal medium by shaking with 8-hydroxyquinoline. Other conditions were as described in Table 1.

	Riboflavin [% of that produced on $(NH_4)_2SO_4$]	
Nitrogen source	Before removal of iron	After removal of iron
$(NH_4)_2SO_4$	100	100
DL-Serine	79	250
L-Asparagine	22	36
DL-Glutamic acid	21	23
DL-Ornithine	92	236
DL-Threonine	12	8
DL-Citrulline	66	7

DISCUSSION

Two types of experiments with *E. ashbyii* and *A. gossypii* point to purines or purine-like compounds as the precursors of rings B and C of riboflavin (see Goodwin, 1959). With isotopically labelled compounds it has been shown that the labelling pattern of rings B and C is the same as that in the purines (Plaut, 1954*a*, *b*), and in feeding experiments all purines stimulate flavinogenesis to varying extents. Serine and threonine are also stimulatory, the former by providing both formate and 'glycine' (Goodwin & Jones, 1956), and the latter by providing 'glycine' (T. W. Goodwin & A. A. Horton, unpublished observations). As glycine is non-stimulatory it would appear that *E. ashbyii* cannot activate glycine but that 'active glycine' can arise from the metabolism of serine and threonine.

It is obvious from the present results (Tables 7 and 8) that in *C. flareri* rings B and C can also arise from purines for xanthine, the purine most closely related to riboflavin, and to a lesser extent guanine and uric acid are flavinogenic. The relative inactivity of hypoxanthine in *C. flareri* parallels the results with *E. ashbyii* (Brown *et al.* 1958) and is presumably due to the absence of a xanthine oxidase which could convert it into xanthine. The inactivity of adenine in *C. flareri* contrasts sharply with its effectiveness in *E. ashbyii* (Brown *et al.* 1958). Although it can be assimilated into the cells of *C. flareri* (Table 7) it cannot be converted into a

Table 7. *Effect of various purines on growth and flavinogenesis in* Candida flareri

Basal medium with either 7·5 mg. of $(NH_4)_2SO_4$ N plus 12·5 mg. of purine N/100 ml. or 20 mg. of purine N/100 ml. only. Other conditions were as described in Table 1.

	Purine alone			Purine plus $(NH_4)_2SO_4$	
Purine added	Dry wt. (mg.)	Riboflavin (μg.)		Dry wt. (mg.)	Riboflavin (μg.)
Adenine	No significant growth			58·5	72
Hypoxanthine	No significant growth			43·9	83
Xanthine	116·4	59		66·9	562
Uric acid	69·4*	Trace		89	287
Control A: 7·5 mg. of $(NH_4)_2SO_4$ N; no purine	37·9	77	Control B: 20 mg. of $(NH_4)_2SO_4$ N; no purine	27·0	256

* Solutions were slightly cloudy after centrifuging.

Table 8. *Effect of guanine on growth and flavinogenesis in* Candida flareri

Basal medium with 2·32 mg. of N/100 ml. as $(NH_4)_2SO_4$ alone or as purine alone: or with 9·82 mg. of N/100 ml., as $(NH_4)_2SO_4$ alone or including 2·32 mg. of N/100 ml. as purine. Other conditions were as described in Table 1.

N content of medium		Dry wt. (mg.)	Riboflavin (μg.)
2·32 mg./100 ml.	(*a*) as $(NH_4)_2SO_4$	20·3	19
	(*b*) as guanine	24·7	9·5
9·82 mg./100 ml.	(*a*) as $(NH_4)_2SO_4$	32·9	89
	(*b*) as guanine (2·32 mg. of N) + $(NH_4)_2SO_4$ (7·5 mg. of N)	21·7	218

riboflavin precursor. A possible explanation is that the immediate precursor of riboflavin which is produced in the same way as in *E. ashbyii* cannot be deaminated, for no indication of an adenase converting adenine into hypoxanthine could be obtained. On the other hand, an active adenase is present in *E. ashbyii*.

The stimulatory effect of serine in *E. ashbyii* is reproduced in *C. flareri*, but it is somewhat masked because in the latter serine also stimulates growth. In contrast (*a*) glycine is stimulatory in *C. flareri* but not in *E. ashbyii*, which suggests that the former can activate glycine, and (*b*) threonine is stimulatory in *E. ashbyii* but not in *C. flareri*, which suggests that the latter does not possess an enzyme, possibly threonine aldolase, which can cleave threonine to glycine.

There are, however, a group of nitrogenous compounds, urea, arginine and ornithine, which are strongly flavinogenic in *C. flareri* but are quite inactive in *E. ashbyii*. This suggests that in *C. flareri* there is a route of riboflavin synthesis quite distinct from the usual purine pathway. It may involve synthesis of a purine precursor via an unconventional pathway or the direct synthesis of a pyrimidine-like precursor. If the latter is correct the compound formed cannot be any of the conventional pyrimidines, for these are non-stimulatory; however, it is more likely to be a purine because it has already been found that [^{14}C]urea is rapidly incorporated into guanine in the algae *Chlorella pyrenoidosa* and *Scenedesmus basilensis* (Ellner & Steers, 1955). This group of compounds, together with citrulline, are the components of the urea cycle in mammals but it is difficult to devise an explanation based on this metabolic cycle because of the inactivity of citrulline; furthermore a thorough search has failed to demonstrate arginase activity in *C. flareri*. The mechanism of incorporation of these compounds into riboflavin is currently being investigated with isotopes.

SUMMARY

1. On a fully defined medium containing ammonium sulphate as the sole nitrogen source, growth and flavinogenesis in *Candida flareri* are maximal 2–3 days after inoculation. Growth is maximal with 7·5 mg. of ammonium sulphate nitrogen/100 ml. and flavinogenesis maximal at 15 mg. of nitrogen/100 ml.

2. The extreme sensitivity of the flavinogenic system to traces of iron was confirmed; the inhibitory effects of high concentrations of glucose and phosphate were due to the iron present in these compounds.

3. Many amino acids greatly stimulate growth but reduce flavinogenesis; glycine, serine, ornithine, arginine and urea stimulate growth and flavinogenesis.

4. Xanthine, guanine and uric acid as sole nitrogen sources support growth of *C. flareri*; adenine and hypoxanthine do not. In the presence of ammonium sulphate all four stimulate growth, but only xanthine, and to a lesser extent guanine and uric acid, stimulate flavinogenesis.

5. The pyrimidines tested, uracil, thymine, cytosine and orotic acid, neither support growth alone nor stimulate growth or flavinogenesis in the presence of ammonium sulphate.

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Partial Purification and some Properties of a Cellulase from *Helix pomatia*

By FAY L. MYERS and D. H. NORTHCOTE
Department of Biochemistry, University of Cambridge

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The properties of cellulases from *Helix pomatia* have been studied by many workers, including Seilliere (1906), Karrer, Schubert & Wehrli (1925), Karrer & Schubert (1926, 1928) and de Stevens (1955). Cellulolytic enzymes have also been found in other invertebrates, such as *Teredo* (Greenfield, 1955) and *Ctenolepsima lineata* (Lasker & Giese, 1956), and in a wide range of micro-organisms which include some rumen Protozoa (Hungate, 1942) and bacteria (Halliwell, 1957*a*, *b*). Many fungi, for example *Aspergillus oryzae* (Gillespie & Woods, 1953) and *Myrothecium verrucaria* (Saunders, Siu & Genest, 1948; Whitaker, 1953; Miller & Blum, 1956; Grimes, Duncan & Hoppert, 1957), have also been shown to produce cellulases.

Although twofold purifications of *H. pomatia* (de Stevens, 1955) and *Teredo* (Greenfield, 1955) cellulases have been reported, only the cellulase from *M. verrucaria* has been purified to give an electrophoretically homogeneous protein (Whitaker, 1953). The properties of this enzyme have been extensively studied; its molecular weight is 63 000 ± 15 000 (Whitaker, 1952; Basu & Whitaker, 1953; Whitaker, Colvin & Cook, 1954). The enzyme attacks many β-1:4-linked glucosans in an essentially random manner (Whitaker, 1954*a*, *b*, 1956*a*, *b*, 1957; Whitaker & Merler, 1956), although there are deviations from this mode of attack with soluble oligosaccharides. The purified enzyme also attacks wheat-straw xylan (Adams, 1952).

The present work was undertaken to purify cellulase from *H. pomatia*, often a more readily available source than a fungal-culture filtrate, and to compare its properties with those of the enzyme from *M. verrucaria*.

METHODS AND MATERIALS

Preparation of crude extract. Hibernating specimens of *H. pomatia* were dissected by the method of Keilin (1956). The digestive tracts and contents were put into a beaker surrounded by ice. They were cut into 1–2 cm. lengths and the mixture was homogenized with an equal volume of ice-cold water in a cooled homogenizer (Potter & Elvehjem, 1936) fitted with a Perspex pestle and surrounded by ice. The tissue was ground at 2000 rev./min. for periods of 30 sec., at 2 min. intervals, until no large particles could be seen. Usually five such treatments were necessary. The homogenate was centrifuged at 19 000 *g* at 4° for 30 min. and the clear-brown supernatant was dialysed for 16 hr. in cellophan dialysis tubing against a large volume of ice-cold water. Any precipitate which formed was removed by centrifuging at 4° and the resulting solution was used as the starting material from which to purify cellulase. A solution (approx. 100 ml.) containing about 10 mg. of protein N/ml. was obtained from 200 snails.

Preparation of substrates for enzyme assays. At each stage of the purification procedure, the enzymic activity was measured towards cotton linters and Cellofas B.

Bleached cotton linters, kindly given by Lansil Ltd., Shipley, Yorks, were swollen with phosphoric acid for 10 min. at 0° (Walseth, 1952) and the degree of polymerization was estimated by the method of Atchison (1943). The average value was 500.

The degree of substitution of Cellofas B, a soluble carboxymethylcellulose derivative, supplied by Imperial Chemical Industries Ltd. and labelled 'low viscosity grade', was found to be 0·46 by the colorimetric method of Eyler, Klug & Diephuis (1947) and a 0·5% (w/v) solution was prepared. This was stored at 2°.

Methods of cellulase assay. (*a*) Cellofas B as substrate. Cellofas B (5 ml. of 0·5% solution) was mixed with 0·5 ml. of M-buffer (see below) at the required pH and 4 ml. of water in a test tube. The mixture was shaken (50 horizontal strokes of 9·5 cm./min.) in a water bath at 37°. This speed of shaking did not cause any significant loss of enzymic activity (Table 1). Enzyme solution (0·5 ml.) was added and the mixture incubated for 20 min. A sample (2 ml.) was then withdrawn, added to 2 ml. of alkaline copper reagent (Somogyi, 1952) and the mixture heated for 15 min. on a boiling-water bath. It was then cooled rapidly to room temperature, 2 ml. of arsenomolybdate reagent (Nelson, 1944) added, the volume made up to 20 ml. and the colour intensity at 620 mμ read on a Unicam colorimeter (model SP. 350) against a blank with boiled enzyme incubated in the same way. The amount of reducing sugar in the sample

Table 1. *Effect of shaking on cellulase activities*

Crude digestive-tract extract was used as the source of cellulase and the incubation periods were 20 min. for soluble-substrate assays and 17 hr. when cotton linters were used.

Duration of shaking	Specific activity: Cellofas B	Specific activity: Cotton linters
None	260	210
First half of incubation period	300	220
Second half of incubation period	276	190
Whole incubation period	300	200

was then calculated from a calibration curve prepared from glucose (50–200 μg.). Under these conditions the amount of hydrolysis was proportional to the enzyme concentration (Fig 1).

(*b*) Cotton linters as substrate. Oven-dried-bleached and phosphoric acid-swollen cotton linters (0·2 g.) were weighed into a 20 ml. screw-capped bottle. M-Buffer (1 ml.) of the required pH was added, followed by water to give a final volume of 20 ml. The mixture was incubated at 25° with shaking (60 horizontal strokes of 4 cm./min.) for 5 hr. and 1 ml. of enzyme added. The shaking did not cause any fall in the activity of the enzyme (Table 1). After 17 hr., 2 ml. of the solution was withdrawn and the assay completed in the way already described for Cellofas B.

Units of activity. (*a*) For the soluble-substrate assays, one cellulase unit was defined as the quantity of enzyme which gave 1 μg. of glucose when incubated for 20 min. at 37° in a reaction medium (pH 5·6, 10 ml.) containing 0·025 g. of Cellofas B.

(*b*) For the insoluble-substrate assays, one cellulase unit was defined as the quantity of enzyme which gave 1 μg. of glucose when incubated for 17 hr. at 25° in a reaction medium (pH 5·6, 20 ml.) containing 0·2 g. of swollen bleached cotton linters.

(*c*) Specific activity was defined as the units of activity per ml. of the cellulase preparation divided by the extinction of the solution at 280 mμ, measured in a light path of 1 cm.

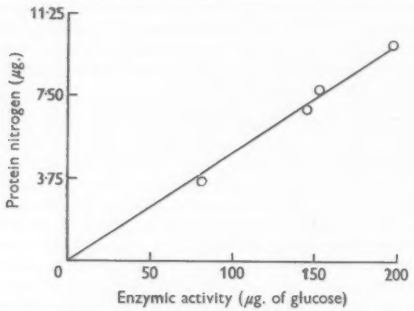

Fig. 1. Variation of cellulase activity with enzyme concentration. Activities were measured towards Cellofas B as described in the Methods section.

Ultraviolet-absorption measurements. These were made in a Beckman DU spectrophotometer or a Unicam SP. 500 spectrophotometer.

Nitrogen. Total nitrogen was estimated by micro-Kjeldahl digestion as described by Chibnall, Rees & Williams (1943), followed by distillation and titration, and ammonia nitrogen was estimated by the method of Conway & Byrne (1937).

Buffer solutions. These were prepared from AnalaR reagents. Except where otherwise stated, the buffers used were phosphate from pH 8·0 to 5·6, acetate from pH 5·6 to 3·5 and glycine–HCl at lower pH values (Gomori, 1955).

pH *Measurements.* These were made with a Pye pH meter and a glass electrode at room temperature, and by standardizing the apparatus with standard buffer tablets, pH values 4·01 and 6·99 (Burroughs, Wellcome and Co.).

Dialysis. Cellophan dialysis tubing (Visking seamless cellulose tubing, Hudes Merchandizing Co. Ltd., London, W. 1) was used wherever possible. When it was necessary to dialyse cellulase preparations for long periods, enzyme preparations in cellophan dialysis tubing 2 cm. in diameter were enclosed in tubing 4 cm. in diameter and then dialysed against a large volume of water. On other occasions, collodion membranes, kindly prepared by Dr G. S. Adair, or pieces of goldbeater's skin (supplied by Frederick Puckridge and Nephew Ltd., London, E. 17), stretched over inverted thistle funnels and attached to them with collodion were used.

Purification of cellulase

H. pomatia cellulase was among the most heat- and acid-labile proteins in the digestive-tract extract; it was not adsorbed on to calcium phosphate gel or aluminium metahydroxide under a variety of conditions; it denatured readily in the presence of ethanol or acetone; it precipitated, with other proteins, over a wide range of ammonium sulphate concentrations.

The first stage of the purification procedure (Table 2) was adapted from that used by Keilin (1956) in the purification of helicorubin from *H. pomatia*.

Stage 1. The crude extract (150 ml.) at 0° was made alkaline to litmus by the addition of aq. 10% (v/v) ammonia solution (sp.gr. 0·88), and ice-cold basic lead acetate solution (25%, w/w) was added to give maximum precipitation, further drops of ammonia being added as required to keep the mixture alkaline. The thick precipitate was removed by centrifuging at 4° and 19 000 *g* for 30 min. and all subsequent operations were carried out at

Table 2. *Purification of* Helix pomatia *cellulase*

Activities were measured towards Cellofas B. Overall purification was fivefold. Overall yield, 4%.

Stage past	Method	Volume (ml.)	10^{-3} × Total activity (cellulase units)	Specific activity
	Initial extract dialysed	150	10 650	445
1	Lead acetate-precipitation and dialysis	90	2 125	995
2	Cellulose column			
	(*a*) Dark-red fractions	60	180	110
	(*b*) Pale-brown fractions	8	2 000	2600
3	IRC 50 column	3	420	2350

this temperature. The pH of the solution was adjusted to pH 6·0 by the addition of M-acetic acid and cold, saturated sodium sulphate solution was added to precipitate excess of lead. After 1 hr. the mixture was centrifuged and 270 ml. of clear-red solution obtained. Solid ammonium sulphate was added until the solution was 80% saturated with respect to ammonium sulphate. A bright-pink precipitate formed, which was removed by centrifuging at 19 000 *g*. It was dissolved in the minimum amount of water, giving 50 ml. of a dark-brown solution. This was dialysed against four changes of 5 l. of water for 16 hr. and was then free from ammonia. The final volume was 90 ml.

Stage 2. Whatman cellulose powder was packed into a column 49 cm. high × 5 cm. diameter and equilibrated in M-acetate buffer, pH 6·0. Material from stage 1 (90 ml.) was adsorbed on to this column and it was eluted at the rate of 60 ml./hr. with the acetate buffer. Fractions (25 ml.) were collected, and immediately after the hold-up volume of the column had passed through, brown fractions were obtained (225 ml.). A colourless solution (1·2 l.) was then obtained. The protein content of each fraction was estimated from the absorption at 280 mμ and the cellulase activities of the fractions were measured. The results (Fig. 2) show that the total and specific activities of the first, coloured fractions were low and that the later fractions contained over 50% of the cellulolytic activity adsorbed on to the cellulose. Fractions 1 to 13 and 14 to 62 were each combined, dialysed against 5 l. of 10% (w/v) ammonium sulphate solution for 16 hr. and any precipitate was removed by centrifuging. The solutions were then dialysed against 10 l. of saturated ammonium sulphate solution for 16 hr. The precipitates which formed were removed by centrifuging at 15 000 *g* for 30 min., redissolved in the minimum volumes of water and dialysed against several changes of water until they were free from ammonia.

A dark-red solution (60 ml.) was obtained from the first fractions and 25 ml. of pale-brown solution from the later ones. The latter solution was frozen at −15° and freeze-dried to a powder, which was redissolved in 8 ml. of water.

Stage 3. Amberlite IRC 50 resin was ground to pass a 200-mesh sieve, washed and converted into the Na^+ form by the method described by Hirs, Moore & Stein (1953). The resin was stirred in water to which 1 ml. portions of 85% (v/v) phosphoric acid were added until the pH of the solution was about 8. The resin was then poured into a glass tube fitted with a no. 3 sintered-glass disk, to give a column 33 cm. high × 2·3 cm. diameter, a disk of glass-fibre paper was placed on the column and the resin was washed with 0·05 M-phosphate buffer, pH 7·05, until the solution eluted was at this pH. The pale-brown solution from the previous stage (8 ml.) was adsorbed on to the column. Fractions (2·2 ml.) were collected and the protein contents and cellulase activities of each are shown in Fig. 3. Fractions 15 to 23 contained the highest total and specific cellulase activity and these fractions were combined and dialysed against saturated ammonium sulphate solution for 16 hr. The precipitate which formed was removed by centrifuging and was dialysed against veronal buffer, pH 7·8, *I* 0·2. It was then examined in a Tiselius electrophoresis apparatus (Perkin–Elmer model 38) against the same buffer. Two boundaries were seen in both the ascending and descending limb of the apparatus.

In the descending limb these had the mobilities $0{\cdot}307 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$ and $1{\cdot}078 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$.

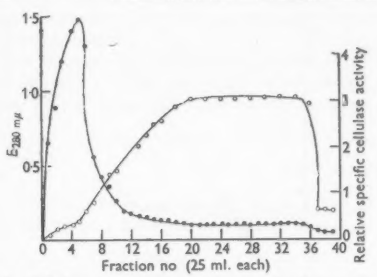

Fig. 2. Cellulose-column fractionation of cellulase (stage 2 of the purification). ●, $E_{280\ m\mu}$; ○, relative activity of cellulase (activity of cellulase adsorbed on to the column was taken as unity). Experimental details are given in the text.

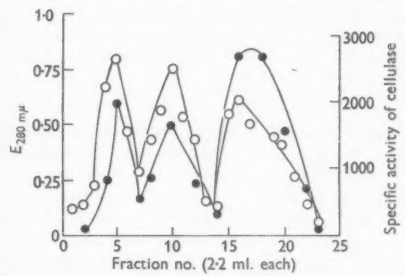

Fig. 3. Fractionation of cellulase on an Amberlite IRC 50 column (stage 3 of the purification of cellulase). ○, $E_{280\ m\mu}$; ●, specific activity of cellulase. Experimental details are given in the text.

RESULTS

Activity of purified cellulase towards carbohydrates. The crude digestive-tract extract breaks down a number of carbohydrates (Myers & Northcote, 1958) and the purified material could degrade some of these, but it was inactive towards methyl β-cellobioside and xylans (Table 3).

Variation of activity with pH. The variation of activity with pH in the crude digestive-tract extract and the cellulase purified past stage 2 is shown in Fig. 4. The same pH optimum, pH 5·6, is found for the soluble and the insoluble substrates in both preparations but the optimum is broader in the crude than in the purified cellulase.

Variation of activity with substrate concentration. The Michaelis constant for the cellulase purified past stage 3 (Table 2) was determined with Cellofas B as the substrate and expressing its concentration in terms of the glucose equivalent. It was found to be 80 mM, by the method of Lineweaver & Burk (1934) (Fig. 5). This may be compared with a value

Table 3. *Activity of purified cellulase towards other carbohydrates*

Methods of assay are given by Myers & Northcote (1958). The enzyme used was purified to the end of stage 2. In the crude extract, the specific activity of the enzyme degrading Cellofas B and cotton linters was 350 and in the purified material 2030. The activity of the crude extract on each substrate is taken as 100 and the relative activity in the purified material thus expresses the enrichment towards each substrate during the purification.

Substrate	pH of assay (pH for optimum activity)	Temperature	Relative activity
Cellofas B	5·6	37°	580
Cotton linters	5·6	25	560
Methyl β-cellobioside	4·75	37	9
Laminarin	5·1	37	1300
Yeast glucan	5·5	25	530
Chitin	4·75	25	0
Xylan	4·5	37	0
Ivory-nut mannan A	5·5	37	470
Ivory-nut mannan B	5·5	25	500

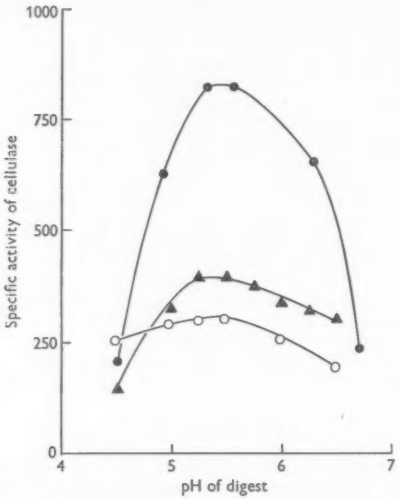

Fig. 4. pH–Activity curves for the crude extract of *H. pomatia* and cellulase purified past stage 2. ●, Purified cellulase, cotton linters as substrate and 0·5 mg. of crystalline bovine serum albumin were added in each assay. ○, Crude extract, cotton linters as substrate. No foreign protein was added. ▲, crude extract, Cellofas B as substrate.

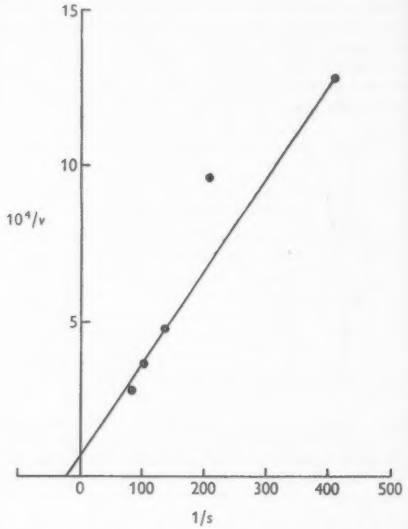

Fig. 5. Lineweaver–Burk plot for determination of K_m on Cellofas B. The enzyme preparation was purified past stage 3; 0·25% Cellofas B solution is 80 mM in terms of its glucose equivalent.

of 4·4 mM found by Jermyn (1952) with an impure cellulase preparation from *Aspergillus oryzae*.

Products of enzyme action. The solution purified past stage 3 (0·5 ml.) was incubated for 16 hr. at 37° with 9·5 ml. of 0·25% Cellofas B in 0·05M-acetate buffer, pH 5·6. The solution was then boiled, centrifuged and the supernatant passed through a column of Amberlite IR 120 (8 cm. × 0·9 cm.) to remove Na^+ ions. The eluate was evaporated under reduced pressure to a thin syrup, which was examined on a paper chromatogram developed in pyridine–ethyl acetate–water (Jermyn & Isherwood, 1949). A spot corresponding to glucose was seen when the paper was coloured with aniline phthalate (Partridge, 1949). A faintly coloured spot near the origin was also seen; this stained yellow when sprayed with bromophenol blue (0·04%) and was due to a carboxymethyl-glucose derivative.

The same cellulase sample (1 ml.) was incubated for 24 hr. at 25° with 19 ml. of a 1% suspension of

cotton linters, the mixture was filtered and the experiment described above repeated. Glucose only was detected chromatographically.

Temperature stability of cellulase. The crude extract was unstable when heated or cooled to very low temperatures (Table 4).

Ammonium sulphate inhibition of cellulase. The results of activity determinations carried out when different concentrations of ammonium sulphate were present in the assay system are given in Table 5. The amount of reducing sugar was calculated in each case from a calibration curve prepared from glucose (25–200 μg.) in the presence of the same concentration of ammonium sulphate as that used in the enzyme assay.

The inhibition was shown to be reversible. The initial extract (2 ml.) was dialysed against 2 l. of 10% (w/v) ammonium sulphate solution for 24 hr. at 4° and a portion of the solution shown to have no activity when assayed in the presence of ammonium sulphate. The remaining solution was then dialysed in a collodion sac against several changes of water until it was salt-free. The specific activity of this solution towards Cellofas B was the same as that of the starting material.

Effect of protein on cellulase activity. The activity of the enzyme purified past stage 2 (Table 2) was greater in the presence than in the absence of

Table 4. *Temperature stability of cellulase*

The initial extract (2 ml. samples) was used. Any precipitate formed during the heating or cooling was removed by centrifuging and the supernatants were diluted for assay.

Treatment	Activity (units/ml.)
Crude digestive-tract extract	7500
Heated at 30° for 5 min.	4000
Heated at 50° for 5 min.	940
Heated at 70° for 5 min.	0
Cooled to −15° and thawed	7500
Cooled to −72° and thawed	0

Table 5. *Inhibition of the initial extract by ammonium sulphate*

The specific activity in the absence of ammonium sulphate was 300.

Concn. of ammonium sulphate (g./l. of assay medium)	Relative cellulase activity
0	[100]
5·5	50
31·2	45
76	40
87·5	0
113	0

Table 6. *Effect of added protein on cellulase activities*

Activities were measured towards bleached, phosphoric acid-swollen cotton linters. The activity of both crude and purified materials in the absence of foreign proteins is taken as 100.

Enzyme preparation	Protein added (mg./20 ml. of assay medium)	Relative activity
Initial extract	Lysozyme (50)	100
	β-Lactoglobulin (50)	100
Cellulase purified past stage 2	Insulin (0·5)	150
	β-Lactoglobulin (0·5)	150
	Crystalline bovine serum albumin (0·5)	150
	Lysozyme (0·5)	145
	Lysozyme (10)	150
	Lysozyme (50)	155

Table 7. *Effect of protein on cellulase activity*

Incubations were carried out at 25°. The added protein was 0·25 mg. of crystalline bovine serum albumin/ml. of incubation mixture.

Duration of incubation (hr.)	Reducing sugar formed during incubation (mg.)			
	Cotton linters (1·0% suspension)		Cellofas B (0·025% solution)	
	Protein absent	Protein present	Protein absent	Protein present
1	0·175	0·18	0·16	0·16
3	0·5	0·54	0·28	0·35
5	1·05	1·22	0·31	0·42
8	1·25	1·6	0·40	0·51
12	2·13	3·04	—	—

protein (Table 6) but the activity of the impure enzyme preparation was not affected by the protein. The extents of the hydrolyses of both soluble and insoluble substrates in the presence of added protein were measured over periods of several hours (Table 7). The effect of pre-incubation on the activity of the enzyme in the absence of its substrate is shown in Table 8.

When the initial extract was used, ivory-nut mannan B, lobster chitin and yeast glucan were degraded more rapidly in the presence than in the absence of added protein (Table 9).

DISCUSSION

M. verrucaria cellulase, the only one purified to electrophoretic homogeneity, has a specific activity only twice as great in the pure solution as in the initial extract. The present work has resulted in the separation of a cellulase with five times the specific activity of the starting material. The purified enzyme is not able to attack xylan or methyl β-cellobioside. It is not electrophoretically homogeneous, however, and is able to degrade a number of carbohydrates other than cellulose. In the purification a cellulose column was particularly useful since it was the only way found to separate the enzyme from contaminating pigments; its success was probably due to interaction between the enzyme and its substrate.

All the three fractions which were separated by the third stage of the purification procedure showed the same order of cellulase activity (Fig. 3). None of these fractions had higher activity than the material adsorbed on to the column, but since the proteins moved at different rates it seems likely that there are at least three cellulolytic enzymes in the digestive tract of *H. pomatia*, possibly differing in their specificities towards the length of glucose chain attacked and the mechanism of the degradation.

Florkin & Lozet (1949) showed that the snail digestive tract harbours cellulose-decomposing bacteria and stated that the cellulase of *H. pomatia* is of bacterial origin: earlier workers (Biedermann & Moritz, 1898; Karrer *et al.* 1925) had assumed that the enzyme was produced by the animal. It is suggested from the present work that since a number of cellulases occur in the digestive tract of *H. pomatia* it is possible that one or more of these are produced by the intestinal flora and others by the animal itself. Tracey (1951) concluded that the cellulase and chitinase of earthworms are of both bacterial and animal origin.

Insoluble substrates are degraded much more slowly than soluble ones, and cellulose is especially inconvenient as a substrate for enzyme assays, since the material is heterogeneous in both its physical and chemical structure and may differ from sample to sample. It was often more convenient therefore to use Cellofas B as the substrate for assays. Only glucose and carboxymethylglucose were found from an enzymic hydrolysate of this substrate, and previous work has shown that carboxymethylcelluloses are degraded in the same way as cellulose (Siu, 1951). Further, the same pH optimum, pH 5·6, was found for cellulase acting on both soluble and insoluble substrates in the present case. However, wherever possible, the results obtained with Cellofas B were confirmed with the insoluble substrate.

Xylans are usually found associated with cellulose preparations and the measured activities of the enzyme could have been partly due to decomposition of these polysaccharides (Halliwell, 1957*a*, *b*). However, during the process of purification the xylanase activity of the original extract (Myers & Northcote, 1958) has been lost and in addition a chromatographic examination of the enzymic hydrolysate of both soluble and insoluble substrates showed the presence of glucose or glucose derivatives only. Preparations of α-cellulose from cotton linters have a very low pentosan content of approximately 0·07% (Adams & Bishop, 1955), and although the original polymer chains have undoubtedly been shortened, especially by the phosphoric acid treatment, the enzymes purified do produce free glucose from these derived glucosans of cellulose and therefore can be defined as cellulases.

Table 8. *Effect of pre-incubation on enzymic activity*

Samples of cellulase (1 ml.) purified past stage 2 and diluted as for assay were pre-incubated as indicated for 17 hr. at 25°. Crystalline bovine serum albumin (2 mg.) was added, as shown in the Table, and activity determinations were made with Cellofas B as the substrate.

Enzyme treatment	Relative activity
Not pre-incubated; no protein added at assay	[100]
Not pre-incubated; protein added at assay	200
Pre-incubated without protein; none added at assay	55
Pre-incubated with protein; none added at assay	190

Table 9. *Effect of added protein on carbohydrase activities towards insoluble substrates*

Assays were carried out at 25° by the methods described by Myers & Northcote (1958). Crystalline bovine serum albumin (5 μg.) was added to each assay mixture.

Substrate	pH for optimum activity	Relative activity (100 in the absence of protein)
Lobster chitin	4·75	215
Ivory-nut mannan B	5·5	350
Yeast glucan	5·5	195

Basu & Pal (1956) reported that a fungal-cellulase preparation was denatured when shaken during assay. However, it can be seen from Table 1 that there was no loss of activity at the speeds of shaking used during assay in the present work. In fact, the tubes were always shaken to ensure both constant-temperature conditions throughout the liquid and thorough mixing of all the components of the system, a particularly important factor when insoluble substrates were used.

Whitaker (1952) and Basu & Whitaker (1953) found that the cellulase activity of a cellulolytic-enzyme preparation from the culture medium of *M. verrucaria*, 40% of the protein of which was cellulase, was increased by the addition of enzymically inactive protein. They presented evidence that the enhancement of activity was due to some effect of the protein on the cellulose substrate, possibly lowering the surface potential. Tracey (1955), working with a highly active chitinase preparation from *Lycoperdon*, found a similar effect which he attributed to the same cause. He suggested that the effect, if confirmed, should be termed an opsonic effect, in contradistinction to the more usual one which is due to the added foreign protein preventing enzyme denaturation. When, therefore, it was found that the activity of a partially purified cellulase preparation from *H. pomatia* was increased by the addition of a number of proteins the possible mechanism of this was further investigated. The evidence reported suggests that the higher activity in the presence of foreign protein is due to the protection of the enzyme from denaturation. This conclusion is based on the following observations:

(*a*) The activity is increased with both soluble and insoluble substrates.

(*b*) The extent of the increase in activity increases with the length of the incubation period (Table 7).

(*c*) A number of proteins with different properties and isoelectric points give the effect (Table 6).

(*d*) Pre-incubation of cellulase with protein in the absence of substrate prevents loss of activity (Table 8).

(*e*) Chitinase, yeast glucanase and ivory-nut mannanase activity in a crude digestive-tract extract from *H. pomatia* were stimulated by the addition of protein with no carbohydrate activity (Table 8). The structures of these polysaccharides differ markedly, as they are made up from different monosaccharide units with different linkages between the units in the molecule, so it is improbable that added protein could have the same effect on each. It should, however, be noted that in these cases the stimulation was observed when the enzymically active solution already contained much inactive protein.

SUMMARY

1. A five-fold purification of a cellulolytic enzyme has been obtained from the digestive tract of *Helix pomatia*. The yield of the enzyme was 4%.

2. Although not specific to cellulose, the purified enzyme did not degrade methyl β-cellobioside or xylans.

3. The purified enzyme showed optimum activity at pH 5·6, was inactivated reversibly by ammonium sulphate and was denatured by heating or cooling.

4. The Michaelis constant of the purified enzyme was 80 mM.

5. The addition of protein to the purified preparation increased its enzymic activity and evidence is presented to indicate that this added protein does not affect the substrate.

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The Transport of Carotenoids, Vitamin A and Cholesterol Across the Intestines of Rats and Chickens

By J. GANGULY,* S. KRISHNAMURTHY and S. MAHADEVAN
Department of Biochemistry, Indian Institute of Science, Bangalore-3, India

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During his classical studies on the isolation and characterization of carotenoids from the tissues of animals, Zechmeister (1937) noticed that different species of animals are able to store different carotenoids selectively. The animals were broadly classified into four groups according to the nature of the carotenoids in their tissues: thus cows have mostly β-carotene, birds mostly hydroxy carotenoids, man and frogs all carotenoids, while most mammals have none. This has subsequently been confirmed by Goodwin (1950) and by Ganguly, Mehl & Deuel (1953). Ganguly, Krinsky, Mehl & Deuel (1952) and Ganguly *et al.* (1953) have postulated that the presence or absence in the tissues of animals of receptor proteins capable of specifically combining with the carotenoid concerned might be the reason for the wide variation in the distribution of carotenoids as related to species. Although Zechmeister & Tuzson (1934, 1935) and Ganguly *et al.* (1953) were able to conclude from the available indirect evidence that this is due to selective absorption in the intestine itself, very little direct evidence was available. Moreover, the exact mechanism and the exact site of selection in the intestine remained obscure.

The selectivity in the absorption of lipids is not confined to carotenoids alone. It has long been known that a similar situation is encountered in the absorption of sterols, since animals do not absorb plant sterols efficiently. Recently Glover & Green (1957) have suggested the possible presence in the intestinal cells of lipoproteins capable of combining stereo-specifically with the particular sterol to facilitate its absorption. By the use of techniques similar to those of Glover & Green (1957) it is demonstrated in the present communication that highly specialized mechanisms exist in the mucosal cells of animal intestines for selective admission of the carotenoids into their tissues.

* Present address: Institute for Enzyme Research, The University of Wisconsin, Madison 5, Wisconsin, U.S.A.

MATERIALS AND METHODS

Light petroleum (b.p. 40–60°; Burmah-Shell) was allowed to stand over an aqueous solution of potassium permanganate for a few days, washed with water, dried over calcium chloride and double-distilled. A.R. acetone was obtained from British Drug Houses Ltd. and A.R. chloroform from Merck. Ethanol was kept over solid potassium hydroxide for 7 days and double-distilled. Antimony trichloride was obtained from Thomas Tyrer Co. Ltd., London; alumina from Merck; Celite 535 from the Johns-Manville Co. Ltd., London; magnesium oxide from British Drug Houses Ltd.; synthetic vitamin A from Hoffman–La-Roche, Basle; and crystalline cholesterol from British Drug Houses Ltd.

β-Carotene. Fresh carrots were minced and saponified at room temperature in brown bottles and exhaustively extracted with light petroleum. The concentrated extracts

were passed twice through alumina columns, from which the β-carotene band was eluted with 4% (v/v) acetone in light petroleum. The solution was finally chromatographed through a magnesium oxide–Celite (1:1) column with 4% (v/v) acetone in light petroleum. The main β-carotene band was eluted from the extruded column with ethanol–light petroleum (1:1) and was finally dissolved in light petroleum. It showed the typical β-carotene spectrum in the same solvent (Fig. 1*a*) and was not purified any further.

Lycopene. This was similarly extracted from saponified tomato pulp and purified by the same procedure. No further purification was attempted and the product gave the typical lycopene spectrum (Fig. 1*b*).

Lutein. This was extracted from saponified fresh maize leaves and was purified as described by Ganguly *et al.* (1952).

All manipulations were carried out in subdued light. The carotenoids were stored in solution in light petroleum in brown bottles in a refrigerator and suitable portions were withdrawn at the time of each experiment. Given amounts of vitamin A or cholesterol and α-tocopherol were dissolved in 10 ml. of light petroleum; refined groundnut oil was then added and the light petroleum evaporated under reduced pressure, the last traces of it being removed in a stream of nitrogen. Similarly, the carotenoids were obtained in solution in fat by adding α-tocopherol and refined groundnut oil to the stock solutions of carotenoids.

Animals. Male rats of this Institute's strain (usually 4–6 months old) were starved for 24 hr., fed with 0·5 ml. of the oily solution of the substance by means of a stomach tube and killed 60–90 min. later by heart puncture. Chickens of both sexes and of unknown breed and weighing about 0·8–1 kg. were obtained from the local market and kept for about 2 weeks on a diet poor in carotenoids, consisting of polished rice, casein and yeast, in order to free the intestines of any carotenoids that might have been present. The birds were then starved for 24 hr., dosed with 2 ml. of oily solution through a stomach tube, and killed by heart puncture 150–180 min. later. Preliminary experiments showed that at these particular intervals the lipids fed were present in the small intestines in sufficient concentrations for active absorption.

Preparation of tissue fractions

The small intestines were immediately removed and the contents washed out with cold physiological saline. The mucosal cells were then scraped off with the blunt end of a scalpel as described by Glover & Green (1957). Both the intestinal muscles (that part of the small intestine left after the removal of the mucosae) and the mucosae were collected in separate beakers previously placed in crushed ice, and weighed. The mucosal cells were then homogenized in 10 vol. of cold 0·25M-sucrose in a Potter–Elvehjem homogenizer. The homogenate, after being filtered through flannel, was fractionated into nuclear, mitochondrial, microsomal and supernatant (cell sap) fractions according to the method of Schneider & Hogeboom (1950) in an International refrigerated centrifuge with a high-speed head attachment. In a preliminary study of the distribution of enzymes among the cell fractions in both species the bulk of the succinic dehydrogenase and alkaline phosphatase activities was located in the mitochondrial and microsomal fractions respectively. No attempt was made to separate the creamy layer, which was added to the supernatant fraction. The homogenate and the cell fractions were made up to volume with 0·25M-sucrose. The whole procedure was carried out at low temperature as quickly as possible.

Extraction of lipids and determination of carotenoids, of free and esterified vitamin A and of free and esterified cholesterol

Suitable portions of the intestinal contents, muscles, whole homogenate and cell fractions were extracted with ethanol and light petroleum in a Waring Blendor as described by Thompson, Ganguly & Kon (1949). The complete spectra in the visible region of the carotenoids were measured in a Beckman spectrophotometer Model DU after chromatography through alumina columns, and their concentrations calculated by using $E^{1\%}_{1cm.}$ values of 2600, 3470 and 2520 for β-carotene, lycopene and lutein at $\lambda_{max.}$ 450–452, 470–472 and 444–446 mμ respectively in light-petroleum solution. Vitamin A ester and vitamin A alcohol

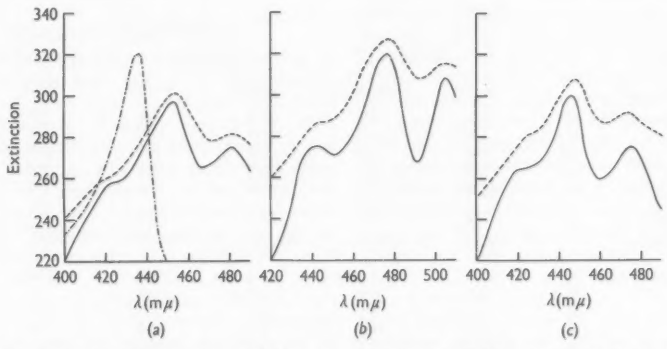

Fig. 1. Spectra of carotenoids [(*a*) β-carotene; (*b*) lycopene; (*c*) lutein] in light petroleum before and after feeding. – – – –, Carotenoids extracted from tissues after feeding; ——, original carotenoids; –·–·–, unidentified pigment in chickens after feeding β-carotene.

and free and esterified cholesterol were determined as described by Krishnamurthy, Seshadri Sastry & Ganguly (1958*b*).

Two species of animals, the rat and the chicken, were chosen. Both convert β-carotene into vitamin A in the intestine, but they differ greatly in their capacity for admitting different carotenoids into their tissues: rats reject all of them, while chickens absorb mainly hydroxy carotenoids. Three types of fat-soluble compounds, namely carotenoids (β-carotene, lycopene and lutein), vitamin A (free, acetate and palmitate) and cholesterol (free, acetate and palmitate), were used. The carotenoids were selected in such a way that one of them (β-carotene) is a hydrocarbon provitamin A, another (lycopene) a hydrocarbon with no provitamin A activity, and the third (lutein) a dihydroxy carotenoid without biological activity. The other two types of fat-soluble compounds (vitamin A and cholesterol) are absorbed by both species of animals. The animals were killed at the time of active absorption after dosing, and the mucosal cells of the small intestines were fractionated by the differential centrifuging procedure of Schneider & Hogeboom (1950). If the lipid fed follows the pathway suggested in Frazer's (1946) hypothesis one would expect to recover it from the supernatant fraction (cell sap) of the cell homogenate. Its presence in the supernatant fraction does not rule out the possibility of its having entered the cell, but if it were found in the particulate materials of the cell this would indicate that during absorption it is transported across the cell not in solution in triglycerides.

RESULTS

Absorption of vitamin A and cholesterol

Vitamin A. Irrespective of the form of vitamin A fed both the ester and the alcohol were present in the intestinal contents, mucosae and muscles of both species (Table 1). The supernatant fraction contained most of the ester, whereas the alcohol was distributed more diffusely among the fractions of the mucosal-cell homogenate. The pattern of distribution of the two forms of vitamin A in their livers also is of a similar nature (Krishnamurthy *et al.* 1958*b*; Krinsky & Ganguly, 1952).

Cholesterol. The results obtained on feeding free cholesterol, its acetate or its palmitate are presented in Table 2. Whatever form of cholesterol was fed, it was always present almost exclusively in the free form both in the mucosal cells and in the muscles, while only traces of the ester could be found. The free cholesterol was largely concentrated in the microsomal fractions of the mucosal cells of both species. The intracellular distribution of cholesterol seems to be similar whether liver cells of rats (Schotz, Rice & Alfin-Slater, 1953; Spiro & McKibbin, 1956), or of chickens (Krishnamurthy *et al.* 1958*b*), or the mucosal cells of rabbits (Glover & Green, 1957), rats or of chickens (present study)

Table 1. *Distribution of vitamin A ester and vitamin A alcohol among the muscles and contents and within the mucosal cells of the small intestines of rats and chickens*

For each experiment individual birds were dosed with 2 ml. of oily solutions containing 15 mg. of vitamin A alcohol, acetate or palmitate and 0·5 mg. of α-tocopherol. Individual rats were dosed separately with 0·5 ml. of oily solutions containing 3 mg. of vitamin A alcohol, acetate or palmitate and 0·2 mg. of α-tocopherol. Tissues from three rats were combined for each experiment. Values are expressed as μg./g. and as the mean of three separate experiments. The vitamin A values obtained in the control animals were negligible. H, Whole homogenate; N, nuclear fraction; Mt, mitochondrial fraction; Mc, microsomal fraction; S, supernatant (cell sap) fraction; tr, trace (detectable but too low for estimation).

			Form of vitamin A fed					
			Alcohol		Acetate		Palmitate	
	Fractions		Ester	Alcohol	Ester	Alcohol	Ester	Alcohol
Chicken	Mucosal cells	H	20·0	3·2	31·0	4·4	9·6	1·6
		N	1·9	0·5	2·9	0·6	1·1	tr.
		Mt	2·4	0·6	3·8	0·7	0·5	0·2
		Mc	2·8	1·0	5·4	1·2	0·5	0·5
		S	12·5	1·3	20·0	1·8	7·3	1·0
	Muscles		7·7	1·6	10·1	1·33	8·3	0·8
	Contents		275·0	2200·0	3100·0	285·0	2500·0	90·0
Rat	Mucosal cells	H	17·0	6·2	28·5	11·0	20·0	9·2
		N	1·2	0·8	2·2	0·9	1·0	0·5
		Mt	2·2	1·1	2·5	2·4	1·5	1·0
		Mc	2·4	1·4	3·6	2·6	2·0	1·5
		S	12·0	2·4	20·1	5·4	16·1	5·8
	Muscles		12·0	3·7	15·5	5·9	10·0	3·5
	Contents		630·0	1920·0	1580·0	380·0	1020·0	447·0

Table 2. *Distribution of free and esterified cholesterol among the muscles, contents and within the mucosal cells of the small intestines of rats and chickens*

Individual chickens and rats were used for each separate experiment. The chickens received 80 mg. of free cholesterol, its acetate or palmitate and 0·5 mg. of α-tocopherol in 2 ml. of oil and rats received 10 mg. of the cholesterol and 0·2 mg. of α-tocopherol in 0·5 ml. of oil. The animals not given cholesterol received the oil and tocopherol alone. Values are expressed as mg./g. and as the mean of three separate experiments. For abbreviations see Table 1.

			Form of cholesterol fed							
			None		Free		Acetate		Palmitate	
	Fractions		Free	Ester	Free	Ester	Free	Ester	Free	Ester
Chicken	Mucosal cells	H	2·60	0·15	2·61	0·08	2·60	0·12	2·50	0·14
		N	0·20	tr.	0·42	tr.	0·23	tr.	0·21	tr.
		Mt	0·50	tr.	0·57	tr.	0·51	tr.	0·52	tr.
		Mc	1·50	tr.	1·52	tr.	1·40	tr.	1·50	tr.
		S	0·25	0·13	0·11	0·08	0·45	0·12	0·25	0·13
	Muscles		2·50	0·12	2·95	0·03	2·85	tr.	2·80	0·03
	Contents		tr.	tr.	21·00	48·00	24·00	16·00	31·00	18·00
Rat	Mucosal cells	H	1·55	0·05	1·53	0·16	1·60	0·06	1·62	0·10
		N	0·18	tr.	0·20	tr.	0·10	tr.	0·10	tr.
		Mt	0·35	tr.	0·35	tr.	0·30	tr.	0·40	tr.
		Mc	0·90	tr.	0·85	tr.	0·95	tr.	0·95	tr.
		S	0·11	0·05	0·09	0·13	0·15	0·05	0·10	0·08
	Muscles		1·57	0·04	2·07	0·04	1·95	tr.	1·85	0·03
	Contents		tr.	tr.	3·23	0·37	2·00	0·79	3·01	0·82

are examined. Another striking feature of cholesterol absorption is that its concentration in the mucosal cells remains almost steady after feeding, while that in the intestinal muscles can be increased only with difficulty. Large amounts of both forms of cholesterol could be found in the intestinal contents (in rats about 3 mg. out of 10 mg. fed and in chickens about 40 mg. out of 80 mg. fed) which would prove that sufficient amounts were present to ensure active absorption. The appearance of both forms of cholesterol in the intestinal contents, irrespective of the form fed, would seem to confirm the presence of both hydrolytic and esterifying enzymes in the intestine.

Absorption of carotenoids

In Table 3 the results of administration of β-carotene, lycopene and lutein are summarized. After the feeding of β-carotene only traces were found in the muscles or mucosae of the rat. The small amounts in the mucosae were mostly in the supernatant fraction, while the particulate materials of the cells were essentially free of it. The vitamin A formed from the β-carotene was largely esterified and was distributed within the cells in the same manner as after the administration of preformed vitamin A. These data seem to agree with the observations of Greenberg (1957) that the conversion of carotene in the intestine is probably by an extracellular mechanism, because the β-carotene seems unable to penetrate the mucosal cells. The traces of it found in the supernatant fraction probably came from the lipids mechanically adhering to the intestinal wall after the contents had been washed out.

β-Carotene suffered the same fate in chickens, except that the extracts of the mucosae and of the muscles contained an unidentified pigment that stayed on an alumina column on elution of β-carotene with 4 % (v/v) acetone in light petroleum. This pigment was eluted with 8 % (v/v) ethanol in light petroleum and gave a spectrum different from that of β-carotene with $\lambda_{max.}$ 434 mμ in light petroleum (Fig. 1*a*). Assuming that its extinction values are the same as those of β-carotene in light petroleum, the average concentrations of β-carotene and of this pigment were 2·2 and 0·9 μg./g. respectively in the mucosae, with traces and 0·72 μg./g. respectively in the intestinal muscles.

The distribution of lycopene was similar to that of β-carotene, and no unusual pigment was formed from the lycopene in either species. Lutein, however, gave a completely different picture. In rats its distribution was similar to that of β-carotene or lycopene, but in chickens the lutein was present in large concentrations both in the muscles and in the mucosae. Within the mucosal cells it was concentrated in the mitochondrial and microsomal particles. The lutein of the cells and of the muscles was in the free form [as only 8 % (v/v) ethanol in light petroleum was able to elute it from an alumina column] and neither lutein nor lycopene underwent any appreciable change, since their spectra were identical with those of the original

Table 3. *Distribution of carotenoids among the muscles and contents and within the mucosal cells of the small intestines of rats and chickens*

For each experiment individual birds were dosed with 2 ml. of oily solutions containing 15 mg. of β-carotene, lycopene or lutein and 0·5 mg. of α-tocopherol. Individual rats were dosed separately with 0·5 ml. of oily solutions containing 3 mg. of β-carotene, lycopene or lutein and 0·2 mg. of α-tocopherol. Tissues from three rats were combined for each experiment. Values are expressed as μg./g. and as the mean of three separate experiments. The values for carotenoids in the control animals were nil. Reisolated β-carotene was determined from the 4% acetone fraction after chromatography through an alumina column. For abbreviations see Table 1.

			Substance fed				
				β-Carotene			
	Fractions		β-Carotene	Vitamin A ester	Vitamin A alcohol	Lycopene	Lutein
Chicken	Mucosal cells	H	2·20	1·80	0·60	0·70	20·70
		N	tr.	tr.	tr.	tr.	3·40
		Mt	tr.	tr.	tr.	tr.	5·10
		Mc	tr.	tr.	tr.	tr.	10·00
		S	2·00	1·50	0·50	tr.	3·50
	Muscles		tr.	0·70	tr.	tr.	13·30
	Contents		2400	Not estimated		5875	3483
Rat	Mucosal cells	H	1·00	2·60	0·50	tr.	2·80
		N	tr.	tr.	tr.	tr.	tr.
		Mt	tr.	tr.	tr.	tr.	0·30
		Mc	tr.	tr.	tr.	tr.	0·90
		S	1·00	2·60	0·40	tr.	1·80
	Muscles		tr.	4·00	0·50	tr.	0·90
	Contents		2200	Not estimated		2490	2900

pigments (Fig. 1*b* and *c*). In a separate experiment (not shown in Table 3), where a mixture of lycopene and lutein in equal proportions (8·0 mg. of each) was given to chickens, only lutein was taken up preferentially by the particulate materials of the mucosal cells and it was present in the intestinal muscles also. The concentration of lutein in both the mucosae and muscles of the intestine were the same as those after feeding only 15·0 mg. of lutein. On opening the small intestines of both species after treatment with the carotenoids, in chickens treated with lutein the villi could be seen, even with the naked eye, to be uniformly coloured yellow, whereas in all other cases the coloured areas were only small and scattered. These patches were probably caused by fatty materials, mechanically held and containing the carotenoids in solution.

Stability of carotenoids in isolated intestines

The experiments *in vivo* with the carotenoids described above do not rule out the possibility of preferential destruction of the carotenoids. Experiments *in vitro* were therefore planned to study the relative stability of the three carotenoids in the isolated intestines of the two species. The animals were killed after starving them for 24 hr., and the small intestines were immediately taken out and cut into 20 cm. segments. Into the segments of the rat intestine 0·5 ml. of oil containing 2·0 mg. of β-carotene, lycopene or lutein and 0·2 mg. of α-tocopherol, and into the segments of chicken intestines 1 ml. of oil containing 6·0 mg. of the particular carotenoid and 0·5 mg. of α-tocopherol, were introduced, and they were then incubated for 90 and 180 min. respectively at 37° in physiological saline. The experiment was carried out in triplicate and analysis of the carotenoids after incubation showed that the concentrations had altered only a little or not at all.

DISCUSSION

Vitamin A, cholesterol and carotenoids are all fat-soluble compounds; yet their behaviour during absorption from the intestine does not seem to be identical. Whether vitamin A alcohol, acetate or palmitate was given, it appeared in the intestinal muscles and in the mucosal cells largely as ester, and most of the ester of the cell was recovered from the supernatant fraction of the cell homogenate. This finding is in general agreement with the earlier reports of Thompson *et al.* (1949), Thompson *et al.* (1950) and of Glover, Goodwin & Morton (1948) where it has been demonstrated that vitamin A in the intestinal wall and in mesenteric lymph is mostly esterified.

The picture was quite different with cholesterol. In all the trials where no cholesterol, free cholesterol or its acetate or palmitate was fed, it was

present in the mucosal cells and in the muscles in the free form and was concentrated in the microsomal fraction of the mucosal cells. Also the absolute values of the free cholesterol could not be increased by any appreciable amounts in the tissue fractions after these treatments. Kim & Ivy (1952) believe that only free cholesterol can enter the cell, and according to Glover & Green (1957) free cholesterol is transported across the cell by a mechanism of simultaneous adsorption and desorption on lipoproteins of the cell. Both suggestions seem to be compatible and our results are in agreement with them since, whatever the form fed, cholesterol appeared mostly in the free form in the intestinal tissue fractions and its concentration could hardly be increased even after heavy dosing.

The mechanism of absorption of carotenoids appears to be different from that of vitamin A or of cholesterol. β-Carotene and lycopene were found only in traces in the mucosae and in the intestinal muscles of both species. In the rat, the concentrations of lutein were similar to those of the other two carotenoids, whereas in the chicken it appeared in marked concentrations in the muscles and in the particulate materials such as the mitochondria and the microsomes of the mucosal cells.

It might be argued that the provitamins A are converted into vitamin A during the process of absorption. This cannot, however, always be the case, because cows are known to absorb (unchanged) a provitamin A, β-carotene, in preference to other biologically inert carotenoids (Gillam & Kon, 1940). Moreover, it should follow that inert carotenoids would be absorbed with equal efficiency. But the results of this study demonstrate that, although neither lutein nor lycopene is a provitamin A, only lutein is able to penetrate the mucosal cells of chickens, while neither of them can enter the mucosal cells of the rat. The preferential absorption of lutein by chickens might be due to the presence of the hydroxyl groups offering anchorage. Rats, however, reject this pigment.

Another possibility is that animals possess efficient enzyme systems that selectively destroy the carotenoids; the recent findings of Bieri (1955), however, rule out this possibility: normally rabbit tissues do not contain any carotenoids, whereas chickens contain both hydroxycarotenoids and traces of carotenes. But once β-carotene, dispersed in water with Tween 40 (polyoxyethylene sorbitan monopalmitate), is introduced intravenously into these animals, it continues to circulate in the blood unchanged for a long time (Bieri, 1955). Our experiments *in vitro* on the stability of carotenoids in isolated intestines of rats and chickens have demonstrated also that no such mechanisms for selective destruction exist in their intestines. It would thus appear that selective absorption of one carotenoid from a mixture is not the result of preferential destruction of the others.

Ganguly *et al.* (1952, 1953) had earlier proposed that the species difference in the absorption and deposition of different carotenoids might be due to the presence of specific receptor proteins in animal tissues. More recently Glover & Green (1957) have pointed out that during absorption from the intestine the sterols are thrown out of fat solution, to be absorbed at the molecular level by stereospecific lipoproteins of the mucosal cells. The results presented here indicate that carotenoids also are similarly thrown out of fat solution during absorption. Here again, it is possible that the intestinal cells of the chicken contain stereospecific lipoproteins capable of combining specifically with lutein at the molecular level and thereby allowing its entry into the animal. More compelling evidence in favour of such a mechanism is that after oral administration of the three carotenoids to rats and chickens, the villi of chickens receiving lutein become uniformly yellow, whereas in all the other experiments they remain almost colourless.

There seems to be general agreement on the specificity of association of lipoproteins with various lipid components; as mentioned above, Glover & Green (1957) believe such a phenomenon to obtain in sterols. Krishnamurthy, Mahadevan & Ganguly (1958*a*) have discussed the specific nature of association of vitamin A ester and vitamin A alcohol with different lipoproteins in rat liver. Krinsky, Cornwell & Oncley (1958) have clearly demonstrated that in post-absorptive human blood different lipoproteins are associated with vitamin A ester, vitamin A alcohol and carotenoids, whereas the chylomicra are essentially free of these lipid compounds. They also have emphasized the highly specific nature of the association of the lipoproteins with such compounds. The findings of this study, when considered with those of the Harvard workers, strongly indicate that during absorption from the intestine the lipids under consideration are separated for transport by specific lipoproteins, whereas the triglycerides are absorbed as chylomicra.

Lutein exists in combination with proteins in chicken blood (Ganguly *et al.* 1952). In chicken liver also it is present in a similar combination with lipoproteins, because it can be fully extracted from the liver homogenate with diethyl ether only after protein denaturation (unpublished results). In the present study, the lutein was not fully extractable with diethyl ether from mucosal-cell homogenates unless the protein was denatured by ethanol (results not included in the text). The pattern of intracellular distribution of lutein in both liver and mucosae of chickens seems to be similar. It is

thus possible that a lipoprotein stereo-specific for lutein exists in dynamic equilibrium among the various tissues of chickens and helps in the transport of lutein across the mucosal cells and in its subsequent distribution among the different tissues. Similarly it is suggested that, in the cow, mainly β-carotene is absorbed, because of the presence of a stereo-specific lipoprotein that selectively combines with β-carotene, whereas in most other mammals carotenoids are not absorbed because of the absence of suitable lipoproteins.

SUMMARY

1. Chickens kept on a low-carotenoid diet and normal adult rats were starved for 24 hr., and dosed with oily solutions of vitamin A alcohol, its acetate or palmitate, free cholesterol, its acetate or palmitate, β-carotene, lycopene or lutein. The rats were killed 60–90 min. later and the chickens after 150–180 min. The intestinal contents were washed out and, after being separated from the intestinal muscles, the mucosal cells were homogenized and fractionated into nuclear, mitochondrial, microsomal and supernatant (cell sap) fractions.

2. In all trials with vitamin A the latter was present mostly as ester in both the intestinal muscles and in the mucosal cells, and this ester could be recovered almost completely from the supernatant fraction of the cell homogenate.

3. It was immaterial whether no cholesterol, free cholesterol, its acetate or palmitate was fed; cholesterol was present predominantly as the free compound both in the muscles and in the mucosal cells, and the microsomal fraction of the mucosal cells contained most of the free cholesterol. The concentration of free cholesterol in the microsomal fraction could not be changed, whereas that in the muscles increased slightly after heavy dosing.

4. Except for lutein in chickens, all carotenoids were found only in traces in both intestinal muscles and in mucosae. In chickens lutein was found in high concentration in the intestinal muscles and in the mucosal cells; in the latter it was concentrated in subcellular particles such as mitochondria and microsomes.

5. The role of stereospecific binding by lipoproteins in the absorption, transport and storage of carotenoids is discussed with special reference to species differences in the selective absorption of different carotenoids.

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The Interaction of some Factors Affecting the Kinetics of a Molluscan β-Glucuronidase

By R. I. COX
Department of Veterinary Physiology, University of Sydney, Australia

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Marine molluscs have been reported as convenient and rich sources of β-glucuronidase (Stitch & Halkerston, 1953; Dodgson & Spencer, 1953; Cox, 1957). These β-glucuronidase preparations have been useful for the hydrolysis of urinary steroid glucuronides. In the application of enzyme preparations for such hydrolyses, rapid reactions consistent with optimum yield of the liberated steroids are desired. In determining optimum conditions for hydrolysis, the effects of the factors pH, duration of reaction and enzyme concentration on the rate of hydrolysis are generally studied with one factor at a time being varied; the interactions of factors affecting the hydrolyses have been neglected. Such interactions are known to affect chemical reactions and may be investigated by factorial tests, in which all possible combinations of all chosen levels of each factor under test are investigated, either together or in blocks or fractional replicates. The use of factorial tests and the general desirability of this form of experimentation has been discussed thoroughly by Fisher (1949) and by Cochran & Cox (1957).

The results presented here give some properties of a molluscan β-glucuronidase prepared from the limpet *Cellana tramoserica* (Cox, 1957). Before a study of the optimum conditions for enzymic hydrolysis of steroid conjugates, the hydrolysis of phenolphthalein glucuronide has been studied to determine whether interactions occur between the factors which affect the enzymic hydrolysis. Highly significant interactions have been found.

MATERIALS AND METHODS

Reagents. Acetone A.R. was fractionally distilled before use. Glycine, reagent grade, was obtained from British Drug Houses Ltd. and sodium phenolphthalein glucuronide from L. Light and Co. Trichloroacetic acid, acetic acid and sodium hydroxide were A.R. grade.

β-Glucuronidase. This was prepared from the limpet *Cellana tramoserica* (Cox, 1957) by the procedure of Dodgson & Spencer (1953), in which the visceral humps are homogenized in chilled acetone. Preparations at two stages of purification have been used. The product after the first acetone precipitation (A) contained 2×10^5 β-glucuronidase units/g. This preparation is suitable for use in the enzymic hydrolysis of steroid glucuronides. The enzyme was further purified by reprecipitating the water-soluble fraction with 6 vol. of acetone at 0°, yielding an off-white water-soluble powder (B) with an activity of 3×10^5 β-glucuronidase units/g. These two products correspond to powders A and B respectively of Dodgson & Spencer (1953). Solutions of β-glucuronidase were prepared by grinding 100 mg. of acetone-dried enzyme with 1–2 ml. of water to obtain a paste. The paste was diluted to 20 ml. and filtered through a cotton-wool plug. Dilutions of this concentrated enzyme solution were prepared as required. Fresh solutions were prepared from the enzyme powder for each experiment.

pH *Measurements.* A glass electrode and a Radiometer model 22 pH meter (Radiometer, Copenhagen) were used.

Activity of β-glucuronidase preparations. This was determined by the method of Bernfeld & Fishman (1953). One unit of enzyme liberates 1 μg. of phenolphthalein/hr. from an mM-solution of phenolphthalein glucuronide at pH 4·3 in 0·1 M-acetic acid–sodium acetate buffer at a temperature of 37°.

General procedure for hydrolyses. The hydrolysis of phenolphthalein glucuronide under various conditions was followed by measuring the amount of phenolphthalein liberated (Talalay, Fishman & Huggins, 1946; Bernfeld & Fishman, 1953). Substrate and enzyme solutions were adjusted to the required pH with M-acetic acid before use. Mixtures of 0·10 ml. of substrate solution, 0·40 ml. of 0·1 M-acetic acid–sodium acetate buffer of the required pH and 0·50 ml. of enzyme solution at 37° were incubated in a water bath at 37° ± 0·1 for the required time, generally 1 hr. The reactions were stopped by adding 1·0 ml. of 5% (w/v) trichloroacetic acid solution. After the addition of 2·5 ml. of glycine buffer (Bernfeld & Fishman, 1953) and 1·5 ml. of water the extinction coefficients of the solutions at 552 mμ were measured. Blank estimations were made by incubating the substrate–buffer mixture and enzyme separately for the required time, and adding 1·0 ml. of trichloroacetic acid solution to the latter followed by substrate–buffer mixture. The rest of the procedure was as used for estimations. Blank estimations generally had an extinction coefficient of zero within reading error when compared with water. The final solutions for colorimetry should be pH 10·2. In preparing the glycine buffer the pH of a reaction blank was checked and, if necessary, the glycine buffer adjusted to ensure this value for the final pH of the colorimetric solutions.

Calibration curves relating the extinction coefficient of solutions to concentration of phenolphthalein were obtained by treating 1·0 ml. of aqueous solutions containing 0–60 μg. of phenolphthalein with 1·0 ml. of 5% trichloroacetic acid, 2·5 ml. of glycine buffer and 1·5 ml. of water and determining the extinction coefficient of the mixture at 552 mμ.

RESULTS

Usual experimental method. The effect of pH, substrate concentration, enzyme concentration, duration and temperature of reaction on the rate of hydrolysis of phenolphthalein glucuronide were first investigated in the usual way, varying each factor separately.

With a substrate concentration of mM an optimum for the hydrolysis was at pH 4·2–4·4 (Fig. 1). The effect of substrate concentration on the reaction rate is shown in Fig. 2. Under the conditions used, in particular at pH 4·25, a value for K_m of 0·06 mM was obtained; this value was determined graphically from the plot of substrate concentration/velocity against substrate concentration. The amount of substrate hydrolysed was proportional to enzyme concentration (0·1–0·8 mg./ml.; S.D. of triplicates, 0·92 μg.of phenolphthalein) and to reaction time (0·5–4·0 hr.; S.D. of quadruplicates, 1·5 μg. of phenolphthalein) over the ranges tested. Apart from the variation in enzyme concentration or duration of hydrolysis in these two experiments, the standard conditions used were: substrate concentration, mM; pH 4·25; temperature, 37°; enzyme concentration, 0·2 mg./ml.; duration of hydrolysis, 1 hr. Variation of the temperature at which the hydrolysis was carried out led to an increase in reaction velocity up to about 60°. Thereafter rapid heat-denaturation of the enzyme is evident (Fig. 3).

The factors tested in the above experiments produced effects which are commonly observed for an enzymic reaction. This normality is important in considering the marked interactions which were found between factors in the experiments described below.

Factorial experiment. The effect of the above factors was investigated in an experiment in which

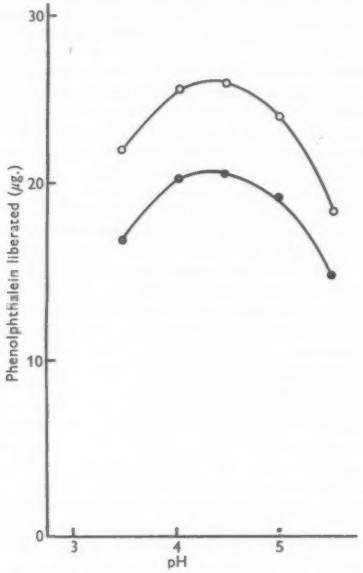

Fig. 1. Effect of pH on the reaction velocity for the hydrolysis of phenolphthalein glucuronide. Substrate concentration mM, hydrolysis for 1 hr. at 37°. Expt. 1, enzyme concentration 0·2 mg./ml. (○); Expt. 2, enzyme concentration 0·16 mg./ml. (●). Each point is the mean of two or three estimations, standard deviation for replicates 0·4 μg. of phenolphthalein.

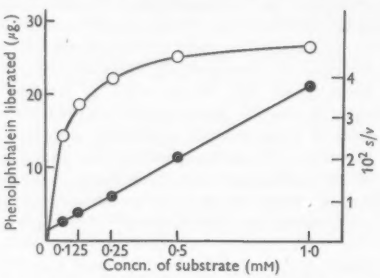

Fig. 2. Variation of reaction velocity with substrate concentration. pH 4·25, enzyme concentration 0·2 mg./ml. and hydrolysis for 1 hr. at 37°. Plot of substrate concentration (s) against velocity (v), ○; plot of s/v against s, ●. Each point is the mean of three estimations, standard deviation 0·4 μg. of phenolphthalein.

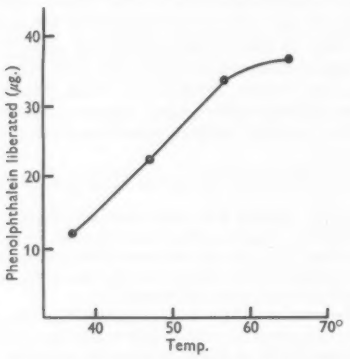

Fig. 3. Amount of phenolphthalein liberated with varying temperature. Substrate concentration mM, pH 4·25, enzyme concentration 0·1 mg./ml. and duration of hydrolysis 1 hr. Standard deviation 0·8 μg. of phenolphthalein for quadruplicates.

all combinations of each factor and level of factor chosen were used. The results of such an experiment may be subjected to an analysis of variance to determine the interactions between factors.

The test was carried out as a 4×2^4 factorial with each of the factors (substrate concentration, enzyme concentration, duration of reaction and temperature) at two levels; four levels of pH were investigated (Table 1). The whole experiment was carried out simultaneously with one batch of reagents. The levels chosen for these factors were within the ranges in which effects of the factors were observed as described in the previous section.

The pH optimum curve shows no significant double curvature ($0{\cdot}3 > P > 0{\cdot}2$) determined from the results of the experiments shown in Fig. 1, i.e. there is no significant cubic component in the regression equation of 'phenolphthalein liberated' on 'pH'. Hence the double-curvature component of pH and the interactions involving it can be used as a measure of error, so avoiding the need to include duplicates in the factorial experiment. As a further check the variance of these components was shown by Bartlett's test to be homogeneous ($\chi^2_{(13)} = 7{\cdot}36$; $0{\cdot}95 > P > 0{\cdot}90$) and not to differ significantly from the variance between duplicates

Table 1. *Summary of analysis of variance: rate of hydrolysis of phenolphthalein glucuronide by β-glucuronidase*

Factor	Levels tested
Temperature (F)	37°, 57°
Time (E)	1 hr., 2 hr.
Enzyme concentration (D)	50, 100 μg./ml.
pH (P)	3·65, 4·25, 4·85, 5·45
Substrate concentration (A)	1·0, 0·1 mM

	Source of variation	Degrees of freedom	Variance ratio	Significance level
Main effects	F	1	1500	***
	E	1	860	***
	D	1	880	***
	P	2	62	***
	A	1	910	***
First-order interactions	EF	1	120	***
	DF	1	142	***
	FP	2	9·5	**
	FA	1	310	***
	ED	1	51	***
	EP	2	4·9	*
	EA	1	160	***
	DP	2	8·0	**
	DA	1	120	***
	PA	2	43	***
Second-order interactions	DEF	1	0·7	—
	PEF	2	0·6	—
	AEF	1	65	***
	PDF	2	2·2	—
	ADF	1	56	***
	APF	2	9·8	**
	PDE	2	1·9	—
	ADE	1	21	***
	APE	2	2·6	—
	APD	2	6·9	**
Third- and fourth-order interactions	PDEF	2	2·0	—
	ADEF	1	8·4	*
	APEF	2	0·8	—
	APDF	2	3·3	—
	APDE	2	1·7	—
	APDEF	2	0·8	—
Error				
Cubic components of *P* and interactions involving it		16	Variance 1·99	

* $P < 0{\cdot}05$. ** $P < 0{\cdot}01$. *** $P < 0{\cdot}001$.

Sources of variation involving *P* are shown with 2 degrees of freedom only as the degree of freedom associated with the cubic components of *P* and interactions involving it are used as error.

in the other experiments carried out with the enzyme (Table 2).

The amounts of phenolphthalein liberated under the conditions used are plotted relative to the pH (Fig. 4). Since only four pH values were tested, only major changes in pH optima are readily apparent; the most marked changes in pH optima are associated with change in substrate concentration. An analysis of variance (Table 1) shows, in addition to the expected significant main-order effects, that all of the first-order and half of the second-order interactions are significant, many at levels where $P<0{\cdot}001$. Thus, for example, the significant second-order interaction of substrate concentration–pH–temperature ($P<0{\cdot}01$) indicates that, within the ranges tested, variation of temperature, substrate concentration or pH affects the interrelation of the other two factors on the hydrolysis rate. For this enzymic reaction and under the conditions used it is important that optimum conditions for the reaction be determined factorially in view of these large interactions.

Further tests. It was decided to investigate in more detail the interaction between substrate concentration and pH. This interaction may be of particular importance in obtaining optimum enzymic hydrolysis of steroid glucuronides, especially as such substrates in urine are commonly present in μM and lower concentrations. It is convenient to consider the interaction in terms of the effect of substrate concentration on the pH optimum. There is a marked shift in the pH optimum in this reaction; at the higher substrate concentrations (mM) an optimum near pH 4·8 was observed whereas at the lower substrate concentration (0·1 mM) no optimum was found within the range tested, although an optimum at a pH lower than 3·6 might be expected.

Table 2. *Bartlett's test for homogeneity of variances*

Source of variation	Degrees of freedom	Variance
Between quadruplicates (Fig. 3; reaction-time and enzyme-concentration experiments)	24	0·747
Between triplicates (Figs. 1 and 2)	30	0·920
Double-curvature components of pH and interactions involving it in factorial experiment (Table 1)	16	1·99

$\chi^2_{(2)}=5{\cdot}39$. $0{\cdot}95>P>0{\cdot}90$.

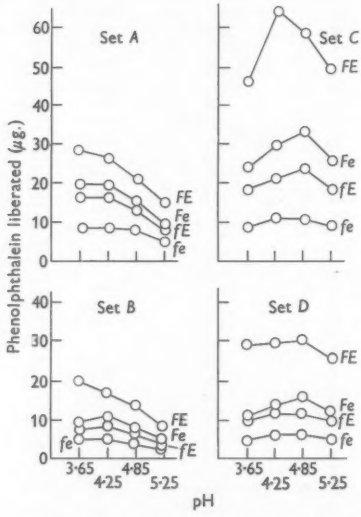

Fig. 4. Results of factorial experiment plotted as effect of pH on reaction velocity. Substrate concentration 0·1 mM for sets *A* and *B*, and mM for sets *C* and *D*. Final enzyme concentration 0·1 mg./ml. for sets *A* and *C*, 0·05 mg./ml. for sets *B* and *D*. Levels of temperature are indicated as *F* (57°), *f* (37°) and duration of hydrolysis as *E* (2 hr.), *e* (1 hr.). Analysis of variance is given in Table 1.

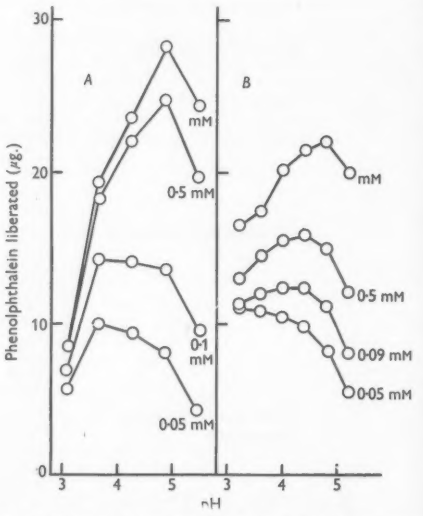

Fig. 5. Effect of phenolphthalein glucuronide concentration on the pH optimum of β-glucuronidase. Substrate concentrations were as indicated, temperature 37° and hydrolysis for 1 hr. *A*, Enzyme preparation (A) at concentration of 0·17 mg./ml. *B*, Enzyme preparation (B) at concentration of 0·08 mg./ml. Standard deviation of replicates 0·4 μg. of phenolphthalein.

Further experiments over a wider range of pH values confirmed the shift of the pH optimum with decrease in substrate concentration. The effect of pH on the liberation of phenolphthalein with substrate concentrations of from 1·0 to 0·05 mM is shown in Fig. 5. Two separate enzyme preparations were used, and also the first (A) was at the usual stage of purification, whereas the other (B) was taken past the second acetone precipitation (see Materials and Methods). Both preparations showed a shift in the pH optimum with change in substrate concentration. Over the pH ranges used (Figs. 4, 5) the enzyme can only be considered to be saturated at the highest substrate concentration, mM. The relation between the submaximum substrate concentration and the shifts in the pH optimum observed is considered in the Discussion.

With preparation (B), a pH optimum lower than 3·05 is indicated at a substrate concentration of 0·05 mM, whereas with (A) at this substrate concentration the pH optimum is near 3·6. Possibly the purer preparation (B) is more stable than (A) at lower pH values.

The variation of pK_m with pH is shown in Fig. 6; the results of two separate experiments are in agreement. The regression of pK_m on pH is a line of slope $-2{\cdot}4$. However, the charge change on desubstration (Dixon, 1953) cannot be derived from this variation of pK_m since the pH optimum is so markedly altered with substrate concentration.

DISCUSSION

The preparation of a crude β-glucuronidase from a marine mollusc *Cellana tramoserica* yields products similar to those obtained from other molluscs such as *Patella vulgata* (Dodgson & Spencer, 1953).

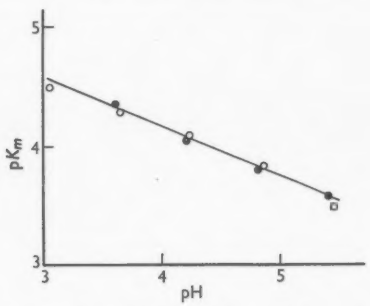

Fig. 6. Plot of pK_m against pH. ●, Values of pK_m determined from the data of Fig. 4; ○, determined from the data of Fig. 5*A*.

The effect of pH, substrate concentration, enzyme concentration, duration and temperature of reaction on the hydrolysis of phenolphthalein glucuronide by this β-glucuronidase follow the pattern generally found with enzymes. From the results with usual procedures, interaction of factors cannot be evident.

The investigation of interactions of these factors showed many highly significant first-, second- and third-order interactions. First-order interactions between pH and temperature, duration of hydrolysis or substrate concentration illustrate the dependence of the 'pH optimum' on the levels of the latter factors. A careful investigation of the action of β-glucuronidase and, in particular, the application of the enzyme to such problems as the hydrolysis of steroid glucuronides should include a survey of interactions between factors; for any particular problem the interactions should be investigated factorially in the working region. Since the β-glucuronidase used showed no abnormal effects in the normal kinetic studies and yet showed significant factor interactions, it is possible that the latter may be of importance for other enzyme reactions.

Factorial experimentation is not necessary for the determination of kinetic constants such as K_m and k_3, which by definition must be investigated under specified conditions. On the other hand, it is noteworthy that factorial experiments may be arranged to give the data obtainable from experiments where one factor at a time is varied, in addition to the information on factor interactions. The interaction between factors can be determined only by factorial experimentation. In studies such as the investigation of optimum conditions for a reaction, consideration of interactions may be essential to the determination of the optimum.

The shift of the pH optimum with substrate concentration change (Figs. 4, 5) is consistent with a requirement for the participation of un-ionized substrate in the enzymic reaction; at the lower substrate concentrations, the concentration of un-ionized substrate may be the limiting factor and decrease of the pH (above the substrate carboxyl group pK of about 3) would increase the un-ionized substrate concentration. In the pH region investigated only the ionization of the carboxyl group of the glucuronyl radical is concerned. If the shift of the pH optimum is due to the dissociation of the substrate, such shifts should be observed with other β-glucuronides suitable as substrates for β-glucuronidase. Where a marked change in pH optimum with change of substrate concentration occurs, it may be possible to hydrolyse preferentially a selected substrate in a mixture of substrates present in different concentrations.

SUMMARY

1. Kinetics of the hydrolysis of phenolphthalein glucuronide by a molluscan β-glucuronidase preparation were investigated, and in particular the interaction of factors affecting the hydrolysis were studied.

2. Highly significant first-, second- and higher-order factor interactions were found, indicating the importance of factorial experimentation in studies on enzyme reactions.

3. Further examination of one interaction, that between pH and substrate concentration, showed a variation of the pH optimum from 4·8 to 3·6 according to the substrate concentration used.

The author is indebted to Professor C. W. Emmens and Dr P. J. Claringbold for extensive advice and guidance in the statistical methods involved. Miss Helen D'Arcy is thanked for her skilled technical assistance. The receipt of a Senior Research Fellowship and grant from the New South Wales Cancer Council is gratefully acknowledged.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **377th Meeting** *of the Society was held at the National Institute for Medical Research, The Ridgeway, Mill Hill, London, N.W. 7 on Friday, 14 November* 1958, *starting at 2 p.m. The following papers were read:*

COMMUNICATIONS

Phospholipid Metabolism in Human Erythrocytes. By C. E. Rowe (introduced by T. S. Work). (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

The *in vitro* incorporation of ^{32}P and $^{14}CH_3COO^-$ into human erythrocyte phospholipids was studied. Whole blood in acid-citrate-dextrose was incubated with the labelled substances at 34·5°.

Both ^{32}P and ^{14}C were incorporated progressively over the course of 10 hr. Chromatography on silica-impregnated paper followed by radioautography indicated that ^{32}P was incorporated into kephalin and lecithin. Chromatography on a silica gel column yielded 250 fractions containing both ^{32}P and ^{14}C.

The fractions were grouped and subjected to methanolysis. The bases obtained were separated by paper chromatography and the methyl esters of the fatty acids examined by gas chromatography. ^{32}P incorporation was greater in phosphatidyl serine than in phosphatidyl ethanolamine or phosphatidyl choline. The degree of acetate incorporation was similar for the different classes of phospholipids. All fractions examined contained different distributions of fatty acids.

The significance of these results with respect to phospholipid synthesis and erythrocyte metabolic activity was discussed.

Biochemical Changes Induced in Krebs II Mouse Ascites Tumour Cells by Infection with Encephalomyocarditis Virus. By J. Malec, E. M. Martin and T. S. Work. (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

The study of biochemical changes induced by virus infection of mammalian cells has been hindered by the lack of suitable host cell preparations in which virus multiplication may be obtained under defined conditions. Sanders, Huppert & Hoskins (1958) have shown that mouse encephalomyocarditis (E.M.C.) virus could be readily propagated *in vitro* in Krebs mouse ascites cells. The ascites cells are easily obtainable in substantial yields and the medium required to support virus propagation *in vitro* is simple. Virus growth may be rapidly estimated by haemagglutination, ascites cell death or plaque-counting techniques. As this system appeared particularly attractive for biochemical studies, investigations were started to determine the effect of virus infection on the protein and nucleic acid metabolism of the host cell.

[^{14}C]Glycine is incorporated into the acid-insoluble proteins of all cytoplasmic components of normal Krebs ascites cells. Infection with E.M.C. virus resulted in increased incorporation into all particulate fractions the increase being greatest in the nuclei and microsomes. A cell-free preparation, comprising ascites cell microsomes, soluble supernatant, added 'pH 5 enzyme' Mg^{2+} ions and phosphoglycerate, incorporated [^{14}C]valine into the microsomal protein. The incorporation of [^{14}C]-valine into microsomes prepared from cells infected with E.M.C. virus occurred at considerably lower rates than into microsomes from uninfected cells. This inhibition was most marked from 3 to 4 hr. after infection, the time at which intracellular virus is first detectable by haemagglutination.

Incorporation of [6-^{14}C]orotic acid into the acid soluble nucleotides and nucleic acids of ascites cells was also studied. The total incorporation into the nucleic acids of infected cells was only 60 % of that of the controls, while incorporation into the acid soluble nucleotides was increased 4 to 5 times after 5 hr. infection with the virus. Thus, these preliminary results indicate that uptake of orotic acid into the cells or its metabolism to mononucleotides is unaffected by virus infection, but its incorporation into the nucleic acids is strongly inhibited.

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The Metabolism of Substituted Thioureas. By R. L. SMITH and R. T. WILLIAMS. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2*)

N-Arylthioureas seem to be far more toxic than *NN'*-diarylthioureas [e.g. LD_{50} for wild Norway rats of *N*-phenylthiourea is 8·6 mg./kg. and of *NN'*-diphenylthiourea is 2000 mg./kg. (Dieke, Allen & Richter, 1947)]. These differences in toxicity could be related to the metabolism of these compounds, but little is known of this aspect, except the report of Carroll & Noble (1949) that, in rats, only small amounts of injected phenylthiourea are excreted as thione compounds.

A number of mono- and di-arylthioureas were fed to rabbits and estimations were made of the urinary excretion of thione (C═S) compounds and various possible conjugates. It was found that the toxic mono-arylthioureas (phenyl-, *p*-chlorophenyl- and *p*-tolyl-thioureas) were desulphurized to a considerable extent (66–100 %) *in vivo*, whereas the relatively non-toxic diarylthioureas (diphenyl-, and 4-hydroxydiphenyl-thioureas) were not (<25 %). The monoaryl derivative, *p*-hydroxyphenylthiourea, is relatively non-toxic and is not extensively desulphurized *in vivo*. Our results suggest that toxicity is related to metabolic loss of sulphur.

The main metabolites of phenylthiourea appear to be phenylurea, which accounts for about 35 % of the dose (40 mg./kg.), and *p*-hydroxyphenylurea (*ca.* 16 %). Paper chromatography indicates that *p*-hydroxyphenylthiourea and phenylcyanamide are also metabolites. Phenylcyanamide is relatively non-toxic and preliminary experiments suggest that *p*-hydroxyphenylurea is one of its metabolites. These results suggest the following mechanism for the metabolism of phenylthiourea:

$$Ph\cdot NH\cdot CS\cdot NH_2 \xrightarrow{-H_2S} Ph\cdot NH\cdot CN \xrightarrow{+H_2O} Ph\cdot NH\cdot CO\cdot NH_2.$$

Toxicity may be related to the release *in vivo* of H_2S which could block iron and copper enzymes.

The main metabolite of diphenylthiourea is the *glucuronide* of 4-hydroxydiphenylthiourea (m.p. 170–171°; Found: C, 51·9; H, 5·1; N, 6·8; S, 7·2; H_2O, 4·4 %. $C_{19}H_{20}O_7NS,H_2O$ requires C, 52·05; H, 5·1; N, 6·4; S, 7·3; H_2O, 4·1 %). 4-Hydroxy- and 4:4'-dihydroxy-diphenylthiourea also form glucuronides which were isolated. 4:4'-Dichlorodiphenylthiourea is not absorbed from the intestine and is eliminated unchanged in the faeces.

This work was supported by Ciba Laboratories.

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The Metabolism of Diaminopimelic Acid and Lysine in Mutants of *Escherichia coli*. By PAULINE MEADOW* and ELIZABETH WORK. (*Biochemistry Department, University College, London, W.C. 1*)

The incorporation has been studied of [^{14}C]lysine or [^{14}C]diaminopimelic acid into cellular fractions of mutants of *Escherichia coli* 9637 requiring one or both of these amino acids for growth in a salts-glucose medium (Davis, 1952; Meadow, Hoare & Work, 1957).

All these mutants and the parent strain incorporated [^{14}C]lysine into their cell walls but not into material soluble in 5 % trichloroacetic acid. Radioautography showed that lysine was the only amino acid labelled after growth in [^{14}C]lysine and unlabelled glucose, although slight radioactivity was detected in an area which was not stained by ninhydrin. Quantitative measurements on the cell walls showed that 88 % of the [^{14}C]lysine in the growth medium was incorporated into lysine and that the diaminopimelic acid was unlabelled.

When the parent strain and diaminopimelic acid-requiring mutants were grown on [^{14}C]diaminopimelic acid and unlabelled glucose and lysine, the only amino acids labelled were diaminopimelic acid and lysine. Quantitative estimations showed that at least half of the cell-wall lysine in these mutants was derived from diaminopimelic acid. When the mutants were grown on [^{14}C]glucose and unlabelled diaminopimelic acid and lysine, the cell walls contained labelled lysine but unlabelled diaminopimelic acid. The specific activity of the lysine extracted from the mutant requiring only diaminopimelic acid for growth was higher than that from the mutant requiring both diaminopimelic acid and lysine.

Fractionation of isolated cell walls with 90 % phenol (Weidel & Primosigh, 1958) showed that both the soluble and insoluble fractions contained diaminopimelic acid and lysine, although most of the diaminopimelic acid was in the insoluble fraction. The specific activities per carbon atom of these two amino acids were similar in both fractions.

The conclusions drawn from these experiments are that extracellular diaminopimelic acid is incorporated into cell walls both by the parent strain and the diaminopimelic acid-requiring mutants and that it is one source of lysine. There is an alter-

* Beit Memorial Fellow.

native route to lysine which does not involve diaminopimelic acid and which is increased by training the diaminopimelic acid- and lysine-requiring mutant to dispense with lysine. There is no conversion of lysine to diaminopimelic acid. From these experiments it is not possible to decide the source of the lysine in the intracellular proteins.

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The Growth-Promoting Activity of Vitamin K and Several Analogues for a Rumen Strain of *Fusiformis nigrescens*. By M. Lev (introduced by H. Gutfreund). (*National Institute for Research in Dairying, Shinfield, Reading, Berks*)

The requirement for vitamin K by a rumen strain of *Fusiformis nigrescens* shown previously (Lev, 1958) suggested the use of this organism to estimate the relative potencies of various forms of this vitamin. The activities of the vitamin K group for *F. nigrescens* are compared with their activity for animals.

The compounds to be tested were made up as a 10 mg./ml. solution in absolute alcohol, and subsequent dilutions were made in distilled water. The medium used was Meat Extract Broth (MEB) consisting of meat extract (Difco) 5 g., Peptone (Difco) 10 g., and NaCl 3 g. per litre of distilled water. The medium was adjusted to pH 7·5 and dispensed in 10 ml. amounts. Since the organism is anaerobic and requires blood, tubes of MEB were heated on a boiling-water bath, cooled and 0·1 ml. of a 1/35 dilution of laked blood was added. The dilution of vitamin K was added and the tubes were seeded with 0·05 ml. of a 2-day culture of *F. nigrescens*. Optical density readings were made after incubation at 39° for 2 days under anaerobic conditions.

1:4-Naphthoquinone, menaphthone, menaphthone bisulphite, 2:3-dimethylnaphthoquinone, vitamins K_1, K_2, K_5 (4-amino-2-methyl-1-naphthol), phthiocol, lapachol and two derivatives of phthiocol were tested. These showed in the main the same relative activity for animals as for *F. nigrescens*. However, there were two major differences in specificity. 1:4-Naphthoquinone is highly active for *F. nigrescens*, but shows no activity for animals, and phthiocol which is active for animals has no activity for *F. nigrescens*. Phthiocol inhibits the growth response of *F. nigrescens* to menaphthone, i.e. it acts as an anti-vitamin K for this micro-organism. Analogues of phthiocol which have no activity for animals (Fieser, Tishler & Sampson, 1941) showed activity for *F. nigrescens* at low concentrations, but became toxic at relatively low levels. Most of the compounds tested became toxic in the range 1–5 μg./ml. Vitamin K_1, K_2 and the water-soluble form of menaphthone (menaphthone bisulphite) were relatively non-toxic.

Experiments are in progress to develop the method described above into a practical microbiological assay for the vitamin K group and to use this organism to study the mode of action of this vitamin.

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An Explanation for the Difference between Mammals and Birds in their Response to Thyroxine and Tri-iodothyronine. By J. R. Tata* and C. J. Shellabarger.† (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

It has been shown by Shellabarger (1955) and Newcomer (1957) that L-thyroxine and 3:5:3′-tri-iodo-L-thyronine have equal potencies in chicken in various biological tests; this result is different from that obtained in most mammals (Barker, 1955). The two- to six-fold difference in potencies of the two hormones in mammals is likely to be due to their different rates of diffusion into tissues on the basis of a 2–4 times shorter biological half-life of tri-iodothyronine (Berson, 1956), and a comparatively lower affinity for serum thyroxine-binding proteins in man (see Robbins & Rall, 1957). It was hence of interest to measure the biological half-lives of the two hormones in the chicken and to compare the thyroxine-binding properties of avian and human sera. The biological half-lives of thyroxine and tri-iodothyronine were found to be

* Beit Memorial Fellow.

† Public Health Service Fellow of U.S. Department of Health, Education and Welfare.

identical (22·5 hr.) in the chicken. It was also found that a thyroxine-binding protein comparable to that present in mammalian serum was absent in chicken and duck blood. Both endogenous and exogenous thyroid hormones are bound to the serum albumin in birds; whole bird serum has a lower affinity for thyroxine than that of human serum and chick and duck serum albumins bind thyroxine and tri-iodothyronine with the same affinity. It is hence concluded that the difference in response to thyroxine and tri-iodothyronine in birds and mammals is due to fundamental differences in the mode of peripheral hormone transport.

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Incorporation of Different Precursors in Conditions of Increased Cholesterol and Fatty Acid Biosynthesis. By R. Paoletti, P. Paoletti and S. Garattini. (*Institute of Pharmacology, University of Milan, Italy*)

Several compounds, [1-^{14}C]acetate, [1-^{14}C]-*n*-butyrate and [2-^{14}C]mevalonic acid, were observed to be incorporated into liver cholesterol and fatty acids with different efficiency. An increase in the incorporation of [1-^{14}C]acetate into cholesterol was shown after total body X-irradiation (Lerner, Warner & Entenman, 1953), after Triton WR 1339 (a polymer of *p-iso*octylpolyoxyethylenephenol) injection (Frantz & Hinkelman, 1955) or during the hepatic regeneration after partial hepatectomy in the rat (Garattini, Paoletti & Paoletti, 1958).

A study has now been made, using the reported precursors in the three experimental conditions of increased cholesterol biosynthesis, extending the observation to fatty acid biosynthesis and substrate oxidation. The experiments were carried out using liver slices incubated in Krebs–Ringer phosphate solution. Cholesterol was isolated as the digitonide, total fatty acids were assayed (James, Lovelock, Webb & Trotter, 1957) and the substrate oxidation determined by $^{14}CO_2$ production (Lyon & Geyer, 1954).

Using livers from X-irradiated Long–Evans male rats (3750 r., 24 hr. before killing) acetate and mevalonic acid are incorporated into cholesterol about five times more efficiently than in the controls; but only acetate is more actively incorporated into fatty acids. Butyrate incorporation into both lipid fractions is unchanged. Cholesterol and fatty acid biosynthesis after Triton injection (200 mg./kg. i.v.) is considerably increased: about sixfold from acetate and twice from mevalonic acid. No change is observed from butyrate.

Cholesterol and fatty acid biosynthesis is increased about sevenfold from butyrate and, to a lesser extent, from acetate, in livers from partially hepatectomized Long–Evans male rats, killed 72 hr. after the operation. No variation is evident from mevalonic acid. On the contrary, substrate oxidation in all reported conditions, is not significantly changed.

The increased utilization of [1-^{14}C]acetate in the experimental conditions described above, may be antagonized by some inhibitors of coenzyme A and particularly by a small series of diphenylacetic acid derivatives as below.

R = ethyl, propyl, butyl, amyl.

The degree of activity of these compounds increases with increasing length of the lateral chain, with about the same progression as previously shown on coenzyme A acetylation assay (Garattini, Morpurgo & Passerini, 1958).

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Nucleotide-Bound Peptides from *Streptococcus faecalis*. By A. D. BROWN. (*Department of Bacteriology, The University, Manchester*)*

In addition to four compounds previously described as 'adenylyl peptides' (Brown, 1958), four more related compounds have now been obtained. Present evidence suggests that all eight compounds are more complex than implied by the earlier nomenclature.

The cell-wall material was removed from the 67% ethanol precipitate as described previously (Brown, 1958) and the latter was dialysed for 4 days against cold water. The previous compounds were located in the dialysate and the present series in the non-dialysable fraction. This was subjected to paper electrophoresis at pH 5·9 (2 v/cm. for 24 hr.) followed by paper chromatography in the ethanol–ammonium acetate solvent (pH 3·8) and u.v.-absorbing materials located. As previously, adenine was the only base which could be detected in these compounds either spectrophotometrically or by chromatography of the free base after hydrolysis. Complete acid hydrolysis liberated a range of amino acids of which glycine, glutamic acid and aspartic acid were predominant and always present. Cysteic acid, serine and alanine were present in varying proportions. Ratios, adenine:amino acid varied over the range (approximate) 1:1 (for glycine in one case) to 10 or 20:1.

* I.C.I. Fellow. Present address: Marine Biological Laboratory, Cronulla, N.S.W., Australia.

Mild acid and alkaline hydrolyses led to the following observations. Peptides and small amounts of free amino acids could be released from the nucleotide moiety by 1 hr. at 100° in 0·1 N-HCl, by 30 min. at 100° in 0·1 N-NH_4OH and by 30 min. at 37° in 0·1 N-NaOH. The first treatment degraded the nucleotide to adenine while the other two produced degradation products in which peptides and/or amino acids were still associated with nucleotides. Hydrolysis for 10 min. at 100° in 0·01 N-HCl and in 0·1 N-HCl produced a spot corresponding to 5′-AMP in the ethanol–ammonium acetate solvent and several other u.v.-absorbing materials, some of which still reacted with ninhydrin.

The evidence is interpreted to mean a polyadenylic acid structure with peptides and possibly individual amino acids attached at different points. It is thought that the stability of the nucleotide-peptide (amino acid) bond under alkaline conditions is greater than would be expected of an acid-anhydride, and it is therefore suggested that ester formation between ribose –OH and amino acid –COOH might be implicated (cf. Hoagland, 1958).

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The Stimulation of Coproporphyrin Formation by an Ultra-Filterable Heat-Stable Factor. By H. HEATH and D. S. HOARE. (*Department of Chemical Pathology, University College Hospital Medical School, London, W.C.* 1)

Whole cell suspensions of *Rhodopseudomonas spheroides* cannot utilize porphobilinogen for the synthesis of porphyrins (Lascelles, 1956) unless they have been previously frozen and thawed (Hoare & Heath, 1958*a*). Removal of the supernatant from a centrifuged frozen and thawed cell suspension, leaves a cell residue with a diminished activity for synthesizing coproporphyrin from porphobilinogen, (Hoare & Heath, 1958*b*) and addition of the supernatant restores the coproporphyrin-forming activity. Better separation is obtained by extracting acetone-powders of whole cells rather than the whole cells themselves. The enzyme preparations thus obtained rapidly lose activity on storage at −15°, but stable preparations can be obtained by extracting with 5×10^{-4} M-BAL (cf. Mauzerall & Granick, 1958, who protected their preparation of uroporphyrinogen decarboxylase with glutathione). If acetone-powders of *R. spheroides* are extracted three times by freezing and thawing, centrifuged, the supernatant ultrafiltered and the extracted residue made into an acetone-powder, one obtains three fractions: (*a*) a soluble non-ultra-filterable residue; (*b*) an ultrafiltrate; (*c*) an acetone-powder of the extracted cells. Under aerobic conditions of incubation all three fractions are necessary for maximum coproporphyrin formation from porphobilinogen. Fraction (*a*) contains the necessary enzymes for the ring closure and decarboxylation; fraction (*b*) contains a stimulating factor which is not affected by boiling in neutral solution; and fraction (*c*) contains another factor which is destroyed on boiling. If the incubation is carried out anaerobically, in the dark however, then only fractions (*a*) and (*b*) are necessary for maximum coproporphyrin formation

and it is concluded that fraction (*c*) contains heat-labile compound(s) which protect uroporphyrinogen from oxidation when incubated aerobically. The ultra-filterable factor is also present in blood, yeast and liver. The factor stimulates when either porphobilinogen or uroporphyrinogen are used as substrates anaerobically. Typical results are: (A) Fraction (*a*), incubated 2 hr. at 37° with 100 μg. porphobilinogen gave 6 μg. coproporphyrin and 28 μg. uroporphyrin; plus fraction (*b*) it gave 22 μg. coproporphyrin and 17 μg. uroporphyrin. (B) Fraction (*a*) incubated 22 hr. at 37° with 75 μg. uroporphyrinogen gave 20 μg. coproporphyrin and 42 μg. uroporphyrin; plus fraction (*b*) it gave 52 μg. coproporphyrin and 10 μg. uroporphyrin.

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Purification of Secretin. By G. G. F. Newton, J. W. Love, N. G. Heatley and E. P. Abraham. (*Sir William Dunn School of Pathology, University of Oxford*)

Recent work on the polypeptide hormone secretin has indicated that it is much more active than had previously been supposed (Jorpes & Mutt, 1955; Legge, Morieson, Rogers & Marginson, 1957). We have also reached this conclusion and have found that highly active material can be prepared in quantity by subjecting crude material to ion-exchange chromatography followed by counter-current distribution between solvents.

Secretin was assayed in rats by the method of Love (1957). The rat unit (r.u.) appears to correspond to 4·4 cat units (Wilander & Ågren, 1932) and to about 0·1 dog units (Ivy, Kloster, Drewyer & Leuth, 1930). Crude secretin hydrochloride* (10–30 r.u./mg., containing 1–3% NaCl) was prepared from pigs' duodena by methods similar in principle to those that have already been described by Friedman & Thomas (1950), Jorpes & Mutt, (1952, 1953) and Gershbein & Krup (1952). The hydrochloride was converted to the acetate by treatment in aqueous solution with Amberlite IRA-400 acetate. The solution of the acetate, adjusted to pH 4·0, was passed into a column of carboxymethyl cellulose (Peterson & Sober, 1956), which had previously been buffered to pH 4·0 with barium acetate. The size of the column was such that it was about 80% saturated with secretin. Elution was carried out first with 0·02N-acetic acid, which removed impurity, and then with 0·1N-acetic acid, which eluted the secretin in a sharp band. Secretin hydrochloride (200–1000 r.u./mg., depending on the quality of the starting material) was prepared from the eluate.

Counter-current distribution of material obtained from carboxymethyl cellulose columns was carried out in two solvent systems. System I consisted of 2-butanol–water–dichloroacetic acid (100:100:1, by vol.) and system II of phenol saturated with water–water saturated with phenol–acetic acid (50:100:5, by vol.). A system similar to I has been used independently by Legge *et al.* (1957). Ninety-five transfers in system I resolved secretin (250 r.u./mg.) into two major active components, A and B, and a minor one, C. The partition coefficients K (=concn. in organic phase/concn. in aqueous phase) for secretins A, B and C were 0·725, 0·125 and 0·018 respectively. Secretin A (4000 r.u./mg.) and secretin B (200 r.u./mg.) were recovered from the distribution fractions as white powders. Distribution curves for secretin activity, dry weight, ninhydrin colour and extinction at 278 mμ showed that secretin B was still grossly impure and that secretin A was intimately mixed with at least two inactive impurities. Considerable improvement in the purity of both active components can be expected by a more extended distribution in this system. Twelve transfers in system II showed that inactive material could readily be separated from a preparation of secretin A (1500 r.u./mg.), which had been obtained from a distribution in system I.

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The Recovery of Brain and Erythrocyte Cholinesterase Activity Following Organophosphate Poisoning. By L. C. Blaber and N. H. Creasey. (*C.D.E.E., Porton Down, near Salisbury, Wilts*)

Davison (1955) suggested that cholinesterase (ChE) activity in animals poisoned with organophosphates recovered *in vivo* by simultaneous synthesis of new enzyme and spontaneous hydrolysis of the inhibited enzyme. Recovery was initially rapid and finally slow, and was attributed to the existence of two types of ChE which formed inhibited enzymes of different stabilities.

Inhibited ChE has since been shown to undergo an ageing process (Jandorf, Michel, Egan & Summerson, 1955; Davies & Green, 1956; Hobbiger, 1955) during which it is converted from a form which can be reactivated by hydroxamic acids or oximes to one which cannot be reactivated.

In the present work the parts played by spontaneous hydrolysis, ageing and new enzyme synthesis in the recovery of sheep erythrocyte and rat brain ChE inhibited *in vivo* with dimethyl and diethyl *p*-nitrophenylphosphates, ethyl pyrophosphate, *iso*propyl methylphosphonofluoridate, or di*iso*propylphosphorofluoridate have been studied.

Recovery of ChE activity *in vivo* occurred in two stages. During the first stage, which was rapid, of short duration and the characteristics of which depended on the organophosphorus compound, spontaneous hydrolysis and ageing occurred simultaneously. Both reactions ceased when only aged inhibited ChE remained. The relative rates of these two processes decided the extent of recovery by spontaneous hydrolysis. This stage was the same *in vivo* as *in vitro*. Thereafter, there was no recovery *in vitro* and the second stage of recovery *in vivo* could be correlated with the appearance of new erythrocytes or the turnover of brain protein and was independent of the nature of the organophosphorus compound.

Erythrocyte and brain ChE activity was estimated by standard methods. These have been criticized by Kewitz & Nachmansohn (1957), who claimed that diethyl *p*-nitrophenylphosphate was partly held extracellularly in poisoned animals and that unless it was removed before the tissues were homogenized would lead to false indications of actual ChE levels. Kewitz & Nachmansohn (1957) therefore dried tissues and extracted them with chloroform prior to homogenization in order to remove free inhibitor. A higher ChE level was thereby obtained than when this procedure was omitted. Our experiments show that this increase in ChE could have been due to spontaneous hydrolysis of the inhibited ChE during the drying and not to removal of extracellular inhibitor. Our ChE measurements are not, therefore, invalidated.

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Effect of Vitamin B_{12} Intake on Response of Human Cyanide Metabolism to Oral Tyrosine. By F. Wokes and Mildred M. E. Barnard. (*Ovaltine Research Laboratories, King's Langley, Herts and South Lodge Hospital, Chase Farm Group, Enfield, Middlesex*)

p-Hydroxyphenylpyruvic acid (PHPP) levels in the serum and urine tend to be higher in 'vegans' (Hill, 1952) living on diets almost free from vitamin B_{12}, especially in 'vegans' with low serum B_{12} levels. Oral administration of tyrosine (*ca.* 58 mg./kg.) increases the PHPP level in the blood (Wokes, Badenoch & Sinclair, 1955) and SCN excretion in the urine during the following few hours, and these increases are particularly marked in 'vegans' (Wokes & Picard, 1955*b*). Metabolic experiments have now been carried out on two normal children (boys) and on two vegetarians. In all the subjects there was a rise in SCN excretion reaching its peak 2–3 hr. after administration of tyrosine which in the vegetarians was followed by a fall to lower levels than before tyrosine administration. The rise in SCN excretion in the vegetarians with low B_{12} intakes was greater than in normal B_{12} intakes, but not as great as in the vegans with still lower B_{12} intakes. These findings will be discussed in relation to the hypothesis of Wokes & Picard 1955*a*) that vitamin B_{12} is linked with the cyanide metabolism in protein synthesis, including possibly methionine.

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A Study of the Mechanism of Penicillin Biosynthesis Using L-Cystinyl-L-[*carboxy*-^{14}C]Valine and L-Cystinyl-D-[*carboxy*-^{14}C]Valine. By H. R. V. Arnstein and D. Morris. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

Previous work has shown that L-cystine, presumably after reduction to L-cysteine, and L-valine are incorporated intact into penicillin (Arnstein, 1957). It has been suggested that the dipeptide cysteinylvaline may function as a precursor in penicillin biosynthesis (Arnstein & Crawhall, 1957); the disulphide form of this peptide, with the valine present as either the L- or D-isomer, labelled with ^{14}C in the carboxyl group, has therefore been synthesized and its possible precursor function studied.

L-Cystinyl-L-[*carboxy*-^{14}C]valine and L-cystinyl-D-[*carboxy*-^{14}C]valine were synthesized from *S*-benzyl-*N*-benzyloxycarbonyl-L-cysteine and D- or L-[*carboxy*-^{14}C]valine ethyl ester using di*cyclo*hexylcarbodiimide (Sheehan & Hess, 1955), with subsequent saponification and reduction with lithium in liquid ammonia. The use of lithium instead of sodium in the reductive process facilitated the removal of salt from the product.

Uptake of both peptides into freshly harvested *Penicillium chrysogenum* WIS 51–20 (Halliday & Arnstein, 1956) was measured by the decrease in radioactivity in the medium, and incorporation of radioactivity into both penicillin and mycelial protein was determined. Similar measurements were made in control experiments substituting an equimolar quantity of L-[*carboxy*-^{14}C]valine for peptide. After 1 hr., 68% of the added L-peptide was taken up, but only 35% of the valine. After 2 hr., the uptake of L-peptide was maximum (88%), but that of valine rose to a similar figure only after a further 2 hr. No measurable uptake of the D-peptide occurred.

To facilitate comparison of the results all figures have been adjusted to 1 μc available inside the cells. The corrected specific radioactivities (μc/g.) of penicillin and protein derived from peptide and valine, respectively, were as follows. After 1 hr. incubation, penicillin, 71·5, 54·5; protein, 0·289, 0·578; after 2 hr., penicillin, 48·1, 50·8; protein, 0·508, 0·918; after 4 hr., penicillin, 25·4, 34·0; protein, 0·467, 1·09.

At all times the penicillin/protein radioactivity ratio was thus higher with peptide as precursor than with valine. Essentially similar ratios were obtained in a second experiment into presence of glutathione which was added in an attempt to reduce the disulphide bond.

If the assumption is valid that labelled valine arising from hydrolysis of peptide inside the cell is free to participate in penicillin and protein synthesis on the same basis as free labelled valine added, the marked difference in penicillin/protein activity ratios between peptide and valine suggests that utilization of peptide for penicillin synthesis is not simply a result of its hydrolysis to free valine.

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Acylative Decarboxylation of 2:4-Dinitrophenylglycine. By J. C. Crawhall and R. W. E. Watts. (*The Medical Unit, St Bartholomew's Hospital, London, E.C.* 1)

In connexion with our studies on the conversion of glycine to oxalate in patients with primary hyperoxaluria, we have fed [1-^{13}C]glycine and isolated urinary oxalate, and free urinary glycine as its 2:4-dinitrophenyl derivative. Combustion of this derivative to obtain CO_2 for mass spectrometric analysis would cause considerable dilution of the enriched carbon atom and possible methods of obtaining the carboxyl carbon atom specifically as CO_2 have been investigated. It was not possible to find oxidative conditions which yield the carboxyl carbon atom without further degradation taking place. Buchanan, Reid, Thomson & Wood (1950) reported that CO_2 is liberated from *N*-phenylglycine by heating with acetic anhydride, we have now applied this reaction to 2:4-dinitrophenylglycine (DNP-glycine).

DNP-glycine (15 mg., 0·062 m-mol.) is converted to its sodium salt, dried and heated under reflux with acetic anhydride (1 ml.) for 30 min. The CO_2 is removed in a stream of nitrogen and collected in traps cooled in liquid oxygen. The volume of gas is then measured and transferred to the mass spectrometer for analysis. A yield of 85% of CO_2 is obtained. The isotope enrichment of this CO_2 is 6% less than that obtained by combustion of the

original glycine. This is probably due to some CO_2 being generated by the reagents. Base-catalysts such as sodium acetate and β-picoline cause much CO_2 to be liberated by the reagents, under our conditions this is reduced to a minimum. This reaction has been successfully applied to other DNP-amino acids including DNP-sarcosine and DNP-proline. The fact that these derivatives decarboxylate readily indicates that a cyclic intermediate, either of the type proposed by Cornforth & Elliott (1950) or by Lawson & Searle (1957), may not be essential for this decarboxylation as these amino acid derivatives contain tertiary amino-groups.

If hippuric acid is treated under the same conditions the yield of CO_2 is poor, but the use of an extra 0·5 molar equivalent of NaOH during formation of the sodium salt increases the yield to 50%.

We are indebted to Professor A. Wormall, F.R.S., for facilities for the mass spectrometric analyses.

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Biosynthesis of Steroids by Human Ovaries. By V. J. O'Donnell and Jean G. McCaig. (*University Department of Biochemistry, Royal Infirmary, Glasgow, Scotland*)

Slices of ovaries from cases of Stein–Leventhal's Syndrome were incubated (3 hr. at 37° under oxygen) in Krebs–Ringer phosphate medium (pH 7·4) containing sodium [1-^{14}C]acetate (0·01 M; specific activity 2 μc./mg.), glucose (0·01 M), nicotinamide (0·04 M), fumarate (0·01 M), and human menopausal gonadotrophin. Histological examination of sections of these ovaries showed the characteristic features described by Stein and Leventhal in their syndrome.

After incubation in each experiment the tissue and medium were extracted with ethyl acetate. The organic residue was then partitioned between heptane and 90% methanol. From the former, cholesterol was isolated via the digitonide and was found to be radioactive in all cases. The methanol-soluble material was extracted with a N-NaOH solution to yield a phenolic fraction. Partitioning of the phenols between benzene and 0·075 M-Na_2HPO_4 separated oestriol from the oestrone-oestradiol fraction (Friedgood, Garst & Haagen-Smit, 1948). Treatment of the latter with Girard's Reagent T separated oestrone from oestradiol (Pincus & Pearlman, 1941). After dilution of each oestrogen fraction with the corresponding carrier oestrogen, oestrone, oestradiol-17β and oestriol were isolated. Repeated crystallizations, formation of derivatives, subsequent hydrolysis of these and crystallization of the free hormone were done in all cases. Constancy in specific activity during such procedures is accepted as a criterion of radiochemical purity. Evidence has been obtained for the biosynthesis of oestrone and oestradiol-17β and of a compound which occurs in the 'oestriol' fraction but which is not oestriol.

Carrier steroids (androst-4-ene-3:17-dione, androsterone, testosterone, progesterone and 17α-hydroxyprogesterone) were added to each of the neutral fractions. These compounds were resolved by paper chromatography in the ligroin-propylene glycol system (Savard, 1953). Subject to the limitations of this technique, tentative evidence has been obtained for the biosynthesis of androst-4-ene-3:17-dione, testosterone and 17α-hydroxyprogesterone.

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The Effect of Short-Chain Fatty Acids on Blood Glucose in Sheep. By R. W. Ash, R. J. Pennington and R. S. Reid. (*Rowett Research Institute, Bucksburn, Aberdeenshire, Scotland*)

Several workers (Schultz & Smith, 1951; Jarrett, Potter & Filsell, 1952; Johnson, 1955; Annison, Hill & Lewis, 1957) have reported that administered propionate and, in most cases, butyrate increased the blood glucose concentration in ruminants; acetate was generally less effective. Kronfeld (1957) found that the effect of butyrate depended upon the initial blood glucose level. Reid (1950) found that propionate, and Potter (1952) that propionate and butyrate, but not acetate, rapidly and markedly increased the blood glucose level in insulinized sheep.

Potter's (1952) experiments were repeated with smaller amounts of fatty acid salt. The administra-

tion of 0·25 m-mole of sodium butyrate/kg. caused increases in blood glucose of up to 40 mg./100 ml. within about 10 min. Equimolar quantities of hexanoate appeared to be even more effective, whilst propionate or DL-β-hydroxybutyrate produced smaller responses. Acetate, succinate and lactate had no appreciable effect. The administration of 0·125 m-mole of glucose/kg. produced increases of less than 20 mg./100 ml.

[*carboxy*-^{14}C]Butyrate (0·25 m-mole/kg.) was injected into insulinized sheep and the ^{14}C content of the blood glucose measured after approx. 5 and 10 min. The results indicated that less than 1% of the additional glucose could have been derived from the butyrate. Negligible amounts of labelled butyrate remained in the blood after 10 min. Similar experiments with [*carboxy*-^{14}C]propionate indicated that only a small proportion of the additional glucose could have been derived from the propionate.

[*carboxy*-^{14}C]Butyrate (0·25 m-mole/kg.) was injected into lambs, which were killed after approx. 10 min. Only about 2% of the injected radioactivity was found in the acid-soluble fraction of the liver. The concentration of radioactivity in the other tissues examined (skeletal muscle, heart and kidney) was roughly the same as in liver.

It is concluded that the rise in blood sugar produced by the fatty acids is not due to gluconeogenesis from the fatty acids.

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Brain Phospholipid Metabolism *in vivo*. By A. N. DAVISON and J. DOBBING. (*Department of Pathology, Guy's Hospital Medical School, London, S.E.* 1)

It has previously been shown (Davison, Dobbing, Morgan, Wajda & Payling Wright, 1958; Davison, Dobbing, Morgan & Payling Wright, 1958) that when incorporated in early life, a number of brain lipids, including certain phospholipids, have a remarkable permanence in the central nervous system. Dawson & Richter's (1950) estimate that in adult mouse brain the observed turnover rate was such that an amount of phosphorus equivalent to the total amount contained in the phospholipid fraction would be exchanged in 70 hr. led us to re-examine the fate of phospholipids along the lines of our former experiments. Thus we have followed the fate of ^{32}P incorporated into young rats during the early stages of their development.

In plasma and in all tissues examined other than the brain of these developing rats, ^{32}P is rapidly incorporated into phospholipid on the day of injection with an almost equally rapid subsequent fall in activity ($t_{0.5}$ = approx. 5 days). In contrast ^{32}P is more slowly incorporated into brain phospholipid, reaches a maximum in about 10 days and falls to only two thirds the maximum value in 70 days. Thus neither the slow rate of incorporation nor the even slower rate of decline support a conclusion that there is rapid turnover, even if consideration be given to the changing levels of acid-soluble ^{32}P.

Our earlier work suggested that once myelin lipids are incorporated into the developing nerve sheath they may remain relatively inert for a long period, even though their turnover can be demonstrated in grey matter (Davison *et al.* 1958). On this hypothesis, it might be expected that in adult animals, brain lipid metabolism takes place mainly in this compartment of the central nervous system. Results presented here, and those of earlier workers will be discussed in terms of this hypothesis.

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DEMONSTRATION

The Estimation of Total Serum Protein Using the Technicon Autoanalyser. By L. J. EVANS and I. D. P. WOOTTON. (*Technicon Instrument Company and Postgraduate Medical School of London, Ducane Road, London, W.* 12)

This apparatus is capable of carrying out a number of biochemical analyses in a completely automatic manner. The method for estimating blood urea on this instrument has been described by Skeggs (1957). An adaptation of the biuret method for total serum protein was demonstrated.

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[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **378th Meeting** *of the Society was held in the Department of Biochemistry and Chemistry, The Medical College of St Bartholomew's Hospital, Charterhouse Square, London, E.C. 1, on Friday, 12 December 1958, at 2 p.m., when the following papers were read:*

COMMUNICATIONS

The Isolation of Two Possible Structural Components from Wool Keratin.

By S. Blackburn. (*Wool Industries Research Association, Torridon, Headingley, Leeds* 6)

The chemical study of an insoluble fibrous protein such as wool often requires some form of degradation to smaller units, and the present paper describes the use of dilute acid for this purpose. Data in the literature indicate that hydrolytic procedures applied to wool can lead to products which differ from the original wool with respect to their content of cystine and other amino acids (Biserte & Pigache, 1951, 1952; Blackburn, 1950; Lindley, 1947).

It has now been found that if wool is treated with 2N-hydrochloric acid at 65° the structure is labilized by partial hydrolysis of peptide linkages, and subsequent extraction with water at an alkaline pH gives an insoluble residue (referred to as component II). Acidification of the alkaline solution with acetic acid precipitates component I. These two substances differ greatly in their amino acid compositions which are now reported, component II having a high content of cystine and proline, component I having a high leucine content. The over-all amino acid pattern of component I is generally similar to that of α-keratose and that of component II to γ-keratose, the fractions derived from wool following oxidation of the disulphide linkages (Corfield, Robson & Skinner, 1958).

It is suggested that α-keratose and component I are derived from the same molecular structure in wool, as are γ-keratose and component II. The linkages joining the two structures to each other are not disulphide bridges; the two can be separated from each other when the keratin structure is labilized sufficiently, as by oxidation or hydrolysis of some of the peptide linkages. Component I may be based on an α-helical structure, while component II may be based on a complex folded non-helical structure. The isolation of two components from wool is in some ways analogous to the isolation of components from another fibrous protein, myosin (Szent-Gyorgy, 1955).

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The Spectrophotometric Determination of Serum Proteins at 210 mμ.

By M. P. Tombs, Felicity Souter and N. F. Maclagan. (*Department of Chemical Pathology, Westminster Hospital Medical School, London, S.W.* 1)

Apart from the well-known peak at 280 mμ, the spectra of proteins show a region of intense absorption below 230 mμ. According to Goldfarb, Saidel & Mosovich (1951) there is a maximum at 190 mμ which is due to the specific absorption of peptide bonds. They were able to show that a number of proteins had similar specific absorptions at 210 mμ, and we have found that measurement at this wavelength offers a rapid and simple method for the estimation of serum proteins.

1/1000 or 1/2000 dilutions of serum in 0·9% (w/v) NaCl give a suitable extinction coefficient in the 0·5–1·5 range. The specific absorption of total serum proteins, measured on forty-one normal and pathological sera was $E^{0.1\%}_{1\,cm.} = 20{\cdot}3 \pm 0{\cdot}17$ at 210 mμ. Porcine albumin (Crookes Laboratories Ltd.) gave the same specific absorption.

With the Hilger Uvispek spectrophotometer certain precautions are necessary at 210 mμ, such as the avoidance of dust on optical surfaces and the choice of a suitable hydrogen lamp. It is advisable to use two different concentrations of a suitable standard protein solution, such as porcine albumin, to check the validity of Beer's Law with each series

of measurements. Although many other substances, such as acetate, carbonate, urates, etc., also absorb strongly at 210 mμ, their specific absorptions and their concentration in serum are negligible as compared with those of the proteins.

Albumin was determined after precipitation of globulins with sodium sulphate and gave results in good agreement with micro-Kjeldahl determinations. Precipitation with ammonium sulphate is preferable for routine use. Measurements have also been successfully made on the eluent from the column chromatography of 0·1 ml. of serum according to the ion-exchange cellulose method of Sober, Gutter, Wyckoff & Peterson (1956), since concentrations of protein as low as 3 μg./ml. can be estimated.

The work was supported by generous grants from the British Empire Cancer Campaign.

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A Ribonucleoprotein Component of *Escherichia coli*. By T. J. Bowen, S. Dagley and J. Sykes. (*Department of Biochemistry, University of Leeds, Leeds* 2)

When cell-free extracts of disintegrated bacteria are examined in the analytical ultracentrifuge, well-defined boundaries are observed that are common to several species and are independent of the methods of disintegration used. During experiments to determine the influence of the environment of the cells on such ultracentrifuge patterns (Dagley & Sykes, 1957) we found that the leading boundary (40s) was absent from extracts of *E. coli* incubated for 1 hr. in phosphate buffer (pH 7, 0·13 M, $I = 0·31$) and that the loss was prevented by addition of $1·6 \times 10^{-3}$ M-$MgSO_4$ to the buffer. At low phosphate concentrations the '40s component' was stable in whole cells but was lost on resuspension in KCl or citrate solutions ($I = 0·31$). This component is a ribonucleoprotein (Schachman, Pardee & Stanier, 1952), and although there is no available evidence to relate its particle size to its functions in the cell, attention may be drawn to the use of high phosphate or citrate concentrations in preparing protoplasts: these procedures favour its disintegration. For given concentrations of phosphate and magnesium ions in cultures, an increase in population of the cells to be disintegrated gave rise to additional material sedimenting close to the 40s component, so that the leading peak developed a 'shoulder'. This appears to be the first stage in dissociation of the 40s component following a reduction of the magnesium ion content per cell.

The isolated 40s component dissociated into components of 29s and 20s when dissolved in 0·13 M-phosphate buffer; and these in turn disintegrated in 0·05 M-citrate. When examined by filter-paper electrophoresis the RNA and protein of the products of disintegration still travelled together as nucleoprotein. A similar observation has been made for the ribonucleoprotein particles from rat liver by Petermann & Hamilton (1958). We obtained evidence for components with coefficients significantly higher than 40s only when the preparations were made in aqueous solutions containing 0·01 M-Mg^{2+} ions and no inorganic phosphate (Tissières & Watson, 1958), conditions which may not normally exist inside whole cells. Our observations on the sensitivity of the 40s component inside cells to the ionic environment outside them suggest that its dissociation products may play a part in metabolism and do not support the view that biological activity is shown only when a critical degree of aggregation is reached.

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The Intracellular Distribution of ^{65}Zn in Normal and Tumour Tissues After its Injection into Mice. By M. E. Bartholomew, R. Tupper and A. Wormall. (*The Department of Biochemistry and Chemistry, Medical College of St Bartholomew's Hospital, London, E.C.* 1)

The intracellular distribution of zinc in mammalian tissues has not been extensively studied. Heath (1949) and Heath & Liquier-Milward (1950) obtained evidence for the association of zinc with nucleoprotein from tumour tissue, but Rosenfeld & Tobias (1951) were unable to detect nucleoprotein-bound zinc. The nuclear, mitochondrial and supernatant fractions of normal rat liver were found by Thiers & Vallee (1957) to contain zinc. A greater uptake of ^{65}Zn by spontaneous mammary tumours compared with normal mammary tissue occurs in female mice injected with the isotope (Tupper, Watts & Wormall, 1955). This led us to study the intracellular distribution of ^{65}Zn in the tumours and livers from such mice.

The animals were killed (in groups of 4 or 5) either 1 or 3 days after injection. The pooled livers or tumours from each group were used for the cell fractionation by the method of Schneider & Hogeboom (1950), and the results recorded here are the means for six separate but similar experiments. The results are given as the following ratio: (^{65}Zn content of fraction/mg. N)/(^{65}Zn content of the whole brei/mg. N).

^{65}Zn was found in nuclear, mitochondrial and supernatant (soluble proteins plus microsomes) fractions of mammary tumours and livers. The tumour cells are characterized by their remarkably uniform distribution of ^{65}Zn between all fractions. Lowest and highest concentrations of ^{65}Zn in the 1-day series of mice were mitochondria ('ratio' of 0·92) and supernatant (1·10) respectively. The lowest concentration in the 3-day series was in the nuclear fraction (0·87) and the highest (1·08) in the mitochondria. The differences between all three fractions were very small.

In the livers the lowest concentration of ^{65}Zn was found in the nuclei and mitochondria, the 'ratios' for the two fractions being respectively 0·43 and 0·42 for the 1-day series, and 0·55 and 0·60 for the 3-day series. The concentration of ^{65}Zn in the liver supernatant fraction was very high, being respectively 1·82 and 1·59, 1 and 3 days after the injection.

Our findings for mouse liver are in agreement with those of Thiers & Vallee (1957) for rat liver.

This work was carried out with the aid of grants from the British Empire Cancer Campaign.

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Oximes of αω-Diquaternaryalkane Salts as Antidotes to Organophosphate Anticholinesterases. By W. K. Berry, D. R. Davies and A. L. Green. (*Chemical Defence Experimental Establishment, Porton Down, Wilts*)

Oximes will restore the activity of phosphorylated cholinesterase and as a result can counteract the lethality of organophosphate anticholinesterases. Until recently the best oxime in both respects was pyridine 2-aldoxime methiodide (PAM) (Davies & Green, 1955; Askew, 1957). Poziomek, Hackley & Steinberg (1958) have since described a new group of oximes which appeared superior to PAM and which were obtained by coupling 2 molecules of pyridine 4-aldoxime with an αω-dibromoalkane. Following this work we have synthesized a group of 30 compounds with the general structure $HO{\cdot}N{:}CH{\cdot}C_5H_4N^+{\cdot}[CH_2]_nR^+,2Br^-$ in which we have varied the position of the oxime group in the pyridine ring, the nature of the second basic group R^+, and the number, n, of methylene groups between the two charged nitrogen atoms. The rate at which these compounds (at 2×10^{-5} M) reactivate cholinesterase inhibited with tetraethyl pyrophosphate (TEPP) correlates well with their ability to save the lives of mice when given subcutaneously at 5 mg./kg. together with 17·4 mg./kg. atropine sulphate 10 min. before subcutaneous TEPP. The best compounds were 1:3-di-(4-hydroxyiminomethylpyridinium)propane dibromide, described by Poziomek *et al.* and 1:6-di-(2-hydroxyiminomethylpyridinium)hexane dibromide. These raised the LD_{50} of TEPP about 100 times compared with only 5 times by PAM, and reactivated the inhibited enzyme about 10 times as rapidly as did PAM. The second oxime group in R^+ is not essential as 1-(4-hydroxyiminomethylpyridinium)-3-pyridiniumpropane dibromide and the corresponding γ-picolinium and *iso*quinolinium compounds all

raised the LD_{50} of TEPP about 40 times. The life-saving properties are also influenced by the number of methylene groups, *n*, but the optimum depends on the position of the oxime groups relative to the charged nitrogen atoms. These new oximes are also superior to PAM in saving the lives of mice poisoned with Sarin, but the improvement is not as dramatic as with TEPP.

The toxicity of the compounds varies with both *n* and R^+ and is unrelated to the therapeutic potency. The two most effective compounds are among the least and most toxic respectively of the whole group.

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Reactivation of Phosphorylated Chymotrypsin. By A. L. GREEN and J. D. NICHOLLS (introduced by D. R. DAVIES). (*Chemical Defence Experimental Establishment, Porton Down, Wilts*)

Organophosphates and certain acylating agents react with chymotrypsin (ChTr) to give an inactive monophosphorylated or monoacylated enzyme. Degradation of the phosphorylated or acylated protein gives small peptides in which the phosphoryl or acyl group is invariably attached to the hydroxylic oxygen of a single serine residue (Oosterbaan & Van Adrichem, 1958; Oosterbaan, Kunst, Van Rotterdam & Cohen, 1958). This evidence, together with the similar dependence on pH of phosphorylation, acylation and substrate hydrolysis, suggests that the initial step in each case is phosphorylation or acylation of a serine hydroxyl catalysed by the imidazole ring of a histidine residue. The activity of the acylated enzyme is readily restored in water at a rate increasing with increasing pH and suggestive of a histidine-catalysed hydrolysis of the acylated serine (Cunningham, 1957; Davies & Green, 1958).

Phosphorylated ChTr is relatively stable in water but has been partially reactivated with hydroxylamine (Cunningham, 1954). We have shown that ChTr inhibited with *iso*propyl methylphosphonofluoridate (Sarin) does slowly recover its activity spontaneously in water but, in contrast to the acylated enzyme, the rate of recovery increases as the pH is lowered. Oximes and hydroxamic acids will produce complete reactivation at a rate proportional to the concentration of the reactivator anion and a positively charged form of the inhibited enzyme with p*K* about 7. These results are inconsistent with a histidine-catalysed hydrolysis of phosphoryl-serine but are partly consistent with hydrolysis of a phosphorylated histidine residue. A structure is proposed for the phosphorylated or acylated active centre of ChTr which will explain these differences in the effect of pH on dephosphorylation and deacylation yet not conflict with the sole isolation of phosphoryl- or acyl-serine on degradation.

On storage, organophosphate-inhibited cholinesterase (ChE) undergoes a slow change from a form which can be reactivated to one which cannot. Phosphorylated ChTr does not change in this way. If this conversion in ChE is due not to migration of the phosphoryl group from histidine to serine (Davies & Green, 1956) but to dealkylation of one of the phosphoryl alkoxyl groups, then a similar mechanism to that proposed for ChTr can be proposed for phosphorylation and dephosphorylation of ChE.

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The Effect of Irradiation on Unsaturated Fatty Acids and Sulphydryl Enzymes. By M. E. CLUBB and E. D. WILLS. (*Department of Biochemistry, Medical College of St Bartholomew's Hospital, London, E.C.* 1)

Recent investigations have focused attention on organic peroxides as possible vital intermediates in the production of biological radiation damage. Thus it has been shown that certain organic peroxides produce effects on some bacteria and bacteriophage which are similar to those caused by radiation (Latarjet, 1956), and that peroxides of fatty acids administered to animals cause toxic effects which, in many respects, closely resemble those caused by radiation (Horgan, Philpot, Porter & Roodyn, 1957). In view of these and related findings, a study of the induction of the formation

of peroxides of unsaturated fatty acids by radiations has been commenced. It has previously been reported (Mead, 1952) that relatively low doses of radiation, e.g. 500 r., caused the formation of peroxides from linoleic acid.

Dilute solutions of linoleic acid in pH 9·0 borate buffer (0·05M) were irradiated by X-rays and electrons from the 15 Mev linear accelerator, using dose rates between 25 r./min. and 400 r./min. for X-rays and $1{\cdot}2 \times 10^5$ r./sec. for electrons. X-rays were used for total doses up to 10 000 r. and electrons for doses from 300 r. to 10^6 r. Peroxide formation, when measured as soon as possible after irradiation, occurred only if the total dose was much larger than that specified by Mead (1952), 100 000 r. or greater. The rate of autoxidation of linoleic acid solution in an oxygen atmosphere at 37° was, however, stimulated by doses greater than 10 000 r. Small quantities of peroxide were produced immediately after the irradiation by small total doses, e.g. 1000 r., providing the dose rate was reduced to 25 r. per min. Similar results have been obtained using emulsions of mixed unsaturated fatty acids from linseed oil.

Irradiation of some sulphydryl enzymes in presence of an emulsion of unsaturated fatty acids has been found to cause a much greater destruction of enzyme activity than either irradiation or the emulsion alone. Thus a 5000 r. irradiation of succinic dehydrogenase in presence of an emulsion of unsaturated fatty acids caused 66% destruction of enzymic activity whilst either treatment alone had no immediate effect. These experiments suggest that radiation may induce destruction of vital –SH groups which is mediated by unsaturated fats.

The authors wish to express their thanks to Professor J. Rotblat and his colleagues of the Physics Department of St Bartholomew's Hospital Medical College for kindly providing the irradiations used in this investigation.

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The Chromatography of Thymus Histones and the Demonstration of Proteinase Activity in the Unfractionated Preparations. By D. M. P. Phillips and E. W. Johns. (*Chester Beatty Research Institute, Fulham Road, London, S.W.* 3)

Calf thymus histones have been fractionated by an improved system on carboxymethylcellulose, the columns being eluted successively with 0·002N-, 0·01N- and 0·02N-HCl. These steps were chosen from gradient elution runs. The 0·002N-HCl removed material with ultraviolet absorption but only 1–2% of the total nitrogen. The 0·01N-HCl eluted the main peak (F 2) and about 80% of the *N*-terminal groups were proline residues. The 0·02N-HCl removed most of the remaining histone (F 3) and about 80% of its end groups were alanine residues. The peak F 2 is probably the same material as is left in the supernatant when histones are fractionated with salt–ammonia (Phillips, 1958), since the quantity, amino acid composition and end groups are similar in both cases. Although the end groups of F 3 and the histones precipitated by salt ammonia are alike, the precipitate weighed more and had a higher arginine content. The discrepancy may lie in the retention by the columns of a small amount of arginine-rich histone.

When the unfractionated histones (calf thymus, rat thymus or calf spleen) were mixed with a solution of *N*-acetyl-L-tyrosine ethyl ester at pH 7–9, the ester was hydrolysed and the reaction was inhibited by 0·001M-di*iso*propyl phosphorofluoridate (DFP). The histones alone at these pH showed a threefold increase in *N*-terminal groups in 7 days, mostly due to lysine, alanine and glycine. In this case 0·0026M-DFP was not completely inhibitory.

This proteolysis probably accounts for the variable end groups (especially lysine) reported earlier (Phillips, 1957, 1958). Crystalline trypsin or chymotrypsin acting on the histones also liberated chiefly lysine, alanine and glycine end groups.

The use of DFP in the preparation of the histones (in the salt washes and during dialysis), or avoidance of dialysis by the use of sulphuric acid (subsequently removed as barium sulphate) for the extraction of deoxynucleoprotein, has given preparations in which over 90% of all end groups were due to proline and alanine. With complete control over degradation during preparation the *N*-terminal groups would probably be entirely proline and alanine residues.

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The Path of Hydrogen in Photosynthesis. By V. Moses* and M. Calvin. (*Radiation Laboratory and Department of Chemistry, University of California, Berkeley 4, California, U.S.A.*)

In an attempt to study the pathway of hydrogen in photosynthesis, the incorporation patterns of tritium from highly radioactive tritium oxide have been examined in *Chlorella* cells in the light and dark. A new apparatus has been designed for use in photosynthesis experiments involving tritium oxide of high specific activity (1 c/ml.). Using the photosynthetic incorporation pattern of labelled carbon dioxide as a criterion for normal metabolic behaviour, little evidence of physiological damage due to radiation was observed in the presence of tritium oxide.

Tritium was found in the same substances as incorporate radiocarbon from labelled carbon dioxide during photosynthesis, although the percentage distribution of tritium among these compounds was quite different from that of ^{14}C. Tritium from tritiated water incubated with *Chlorella* in the light appeared first in the sugar monophosphates, phosphoglyceric acid, aspartic acid, glutamic acid, malic acid and glycollic acid. In the dark these substances were also the ones to become labelled most rapidly, though, compared with light conditions, a much greater percentage of the fixed activity was present in the amino acids. About three times as much tracer was fixed in the light as in the dark.

Glycollic acid appeared to be an important intermediate in hydrogen metabolism as it was labelled in the shortest incubation period studied (5 sec.), and was the most radioactive compound present, at least up to 3 min. of incubation. A scheme for hydrogen transport in photosynthesis involving an alternate oxidation and reduction of glycollic and glyoxylic acids is proposed. The interpretation of biochemical studies with hydrogen isotopes is complex owing to non-specific exchange reactions, and these difficulties will be briefly discussed.

* Present address: Department of Chemical Pathology, King's College Hospital Medical School, London, S.E. 5.

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Magnesium Deficiency and Oxidative Phosphorylation. By R. B. Beechey (*Medical Research Council, Experimental Radiopathology Research Unit, Hammersmith Hospital, London, W. 12*), Nancy Alcock, S. Hanna and I. MacIntyre (*Postgraduate Medical School, London, W. 12*)

We have re-investigated the effect of magnesium deficiency on oxidative phosphorylation using rat-heart sarcosomes isolated by the method of Cleland & Slater (1953). Respiration rates were estimated using a vibrating platinum electrode polarized at $-0{\cdot}6$ v. Phosphorus/oxygen (P/O) ratios were measured by the method of Chance & Williams (1955). The rats were maintained on a diet similar to that described by MacIntyre & Davidsson (1958).

Three experiments were carried out. In Expt. I 100 g. female black and white rats were used. Peripheral vasodilation, prominent in albino rats, was not marked, but low levels of serum magnesium were recorded. P/O ratios associated with the oxidation of glutamate were measured at intervals up to 50 days. In the Expts. II and III 40 g. female and male albino rats were used respectively and maintained on the diet for 11 days. P/O ratios associated with the oxidation of α-oxoglutarate were measured at intervals. In the Expt. III sarcosomes were also isolated by the method described by Vitale, Nakamura & Hegsted (1957).

The P/O ratios were estimated in duplicate and are expressed as: mean (number of determinations) $\pm$ S.E.M. The results were: Expt. I, control 2·63 (7) $\pm 0{\cdot}13$, deficient 2·49 (8) $\pm 0{\cdot}20$, the mean difference was $0{\cdot}14 \pm 0{\cdot}15$; Expts. II and III, Cleland & Slater sarcosomes, control 2·62 (10) $\pm 0{\cdot}23$, deficient 2·59 (8) $\pm 0{\cdot}04$, the mean difference was $0{\cdot}03 \pm 0{\cdot}23$. In Expt. III, three determinations of the P/O ratio, for each group, were made with sarcosomes prepared by the method of Vitale *et al.* (1957). The control values were 2·0, 2·3, 2·0, while the deficient rats gave values of 1·7, 2·4 and 2·1.

These results show that there is no lowering of the P/O ratios in isolated rat-heart sarcosomes in magnesium deficiency. The magnesium content of cardiac muscle was probably normal in those stages of deficiency studied; whether a decrease in P/O ratios occurs in skeletal muscle sarcosomes associated with the combined loss of magnesium and potassium in the later stages of deficiency (MacIntyre & Davidsson, 1958) remains to be determined. Vitale *et al.* (1957) claimed an uncoupling effect on heart sarcosomes within 4 days of deficiency. The differences between these and the present results are probably due to differences in techniques. It seems probable that our results more nearly reflect the situation *in vivo*, in view of the less severe procedures used.

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Detection of $BaS^{16}O_3{}^{18}O$ by Infrared Spectroscopy. By B. Spencer. (*Department of Biochemistry, University of Wales, Cardiff*)

The position of cleavage of ester sulphates by sulphatases has been previously studied by mass spectrographic estimation of the ^{18}O content of carbon dioxide derived from the barium sulphate isolated after enzymic hydrolysis of the substrate in the presence of water containing excess $H_2{}^{18}O$ (Spencer, 1958). The presence of the theoretical amount of ^{18}O in the isolated barium sulphate indicated enzymic rupture of the O—S bond whereas for C—O bond splitting the ^{18}O would be found in the desulphated product.

It can be predicted that substitution of the heavier ^{18}O atom for an ^{16}O in barium sulphate would lead to changes of frequency of vibration in the molecule with consequent shifting of the corresponding infrared absorption peaks to lower wave numbers. To check whether the nature of such changes might offer an alternative method of estimating the ^{18}O content of barium sulphate a specimen of this compound containing 87% $BaS^{16}O_3{}^{18}O$ was prepared by acid hydrolysis of potassium *p*-nitrophenyl sulphate in the presence of water containing 90% atoms excess ^{18}O (cf. Spencer, 1958). Examination of the infrared spectrum of $BaS^{16}O_4$ showed the presence of absorption bands at 981 (ν_1), 1187, 1112, 1076 (ν_3), 637 and 611 cm.$^{-1}$ (ν_4). The ν_2 frequency (451 cm.$^{-1}$) is not shown in the infrared. The $BaS^{16}O_3{}^{18}O$ sample had displaced peaks at 961, 1189, 1095, 1059, 634 and 604 cm.$^{-1}$ while the presence of the $Ba^{16}SO_4$ (13% of the sample) was shown as faint peaks at 1072 (shoulder) and 981 cm.$^{-1}$. These assignments are in good agreement with the Teller-Reddich product rule for XY_4 molecules. Calculation of the product from the isotopic masses gives 0·953 and calculation from the observed frequencies gives 0·954.

Mixtures of $BaS^{16}O_4$ and $BaS^{16}O_3{}^{18}O$ of various composition showed resolution of the respective peaks at 981 and 961 cm.$^{-1}$, but not at the other wavelengths. A method of estimation of $BaS^{16}O_3{}^{18}O$ in barium sulphate has been developed based on absorbance readings at 981 and 961 cm.$^{-1}$ and allows the detection of a lower limit of 3% atoms excess ^{18}O and covers the range up to 87 atom % excess with an average accuracy of $\pm 3\%$. This technique is rather insensitive when compared with the mass spectrographic method (cf. Spencer, 1958) but for the study of the site of cleavage of sulphate esters it offers advantages of speed and simplicity when water with a high content of ^{18}O is available. The application of the infrared method to the study of the mechanism of action of several sulphatases will be discussed.

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The Histidine Residue in the Active Centre of Ribonuclease. By E. A. Barnard and W. D. Stein (introduced by J. F. Danielli). (*Zoology Department, King's College, London, W.C. 2*)

There is evidence (Weil & Seibles, 1955) that one histidine residue in ribonuclease appears to be essential for enzyme activity and can be selectively photoxidized. We have examined the reactivity of ribonuclease towards an alklyating agent, bromoacetic acid. Such studies may give information on relations between protein structure and chemical and catalytic reactivities, since the covalent structure of the ribonuclease molecule is now largely known (Hirs, Stein & Moore, 1958; Anfinsen 1957).

Reaction with bromoacetic acid inhibited ribonuclease. The reagent is known to introduce stable carboxymethyl groups at several sites in proteins (Korman & Clarke, 1956). The inactivation reaction was as great at pH 7 as at pH 9, consistent with attack on histidine. Cytidilic acid, a competitive inhibitor, gave effective protection (at 0·025M) against the inactivation, indicating active centre reaction.

The ultraviolet absorption spectrum of the inactivated protein, in acid and alkali, gave no sign of the spectral characteristics of protein *O*-carboxymethyltyrosine. Reaction with diazotized sulphanilic acid of ribonuclease and of its carboxymethyl derivative gave results showing that only

one histidine residue is blocked in the carboxymethyl protein, at 90% enzymic inhibition.

Using [2-^{14}C]bromoacetic acid, the number of carboxymethyl groups introduced (at pH 7) was determined (after dialysis to constant radioactivity) to be approximately one per molecule of inactivated enzyme. Thus, all the data show that a specific reaction occurs at one, and only one, histidine residue in the active centre.

Ribonuclease contains four histidines. We have used partial hydrolysis to identify the one involved. Ribonuclease and its [^{14}C]carboxymethyl derivative were oxidized with performic acid and degraded using chymotrypsin (Hirs, Moore & Stein, 1956). The peptides obtained were satisfactorily separated by paper electrophoresis and paper chromatography. One peptide present in the control (and reactive towards a diazonium reagent) was absent in the test hydrolysate. One new peptide was identified in the latter (and was negative to a diazonium reagent) and this contained the major part of the ^{14}C-label.

These two peptides were eluted, hydrolysed, and the constituent amino acids identified. These correspond in both the peptide from the control and the labelled new peptide to peptide O-Chy-30 in the nomenclature of Hirs *et al.* (1956). Thus, the histidine concerned is unequivocally identified as that nearest to the *C*-terminal end of the ribonuclease molecule.

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Tumour Regression Obtained by the Use of a Nitrogen-Mustard Derivative after Pre-treatment with its Simple Analogue. By P. J. Barnard and J. F. Danielli. (*Department of Zoology, King's College, London, W.C.* 2)

The effects of a series of enzyme-activated nitrogen mustards (Hebborn & Danielli, 1956) have been studied on the Walker Carcinoma.

Intra-muscular injections of *iso*propyl-(*NN*-di-2′-chloroethyl-4-amino)phenylurethane, a substituted nitrogen mustard, into rats bearing the Walker Carcinoma, caused some growth inhibition of the tumour, but did not give complete regressions. In contrast, when administration of this mustard derivative was preceded by a course of injections of its simple analogue, *iso*propyl-phenyl-urethane, complete regressions of the tumour were obtained.

Further experiments indicating the specificity of this sequence have been performed. When the simple analogue was administered alone there was no inhibitory action on the tumour. When the sequence was reversed, i.e. the mustard was administered first and the analogue later, the effects obtained were no greater than those from a corresponding administration of the mustard alone. When a course of injections of both the mustard and the analogue together was given, the effects produced were similar to the effect of the mustard alone, and were always much less than with the original sequence. Another parallel sequence involving treatment with an unrelated mustard derivative, a substituted phenol, preceded similarly by the urethane analogue treatment, did not show potentiation of the effect of the phenolic mustard in any way.

The results are consistent with the general hypothesis, namely, that a relatively inert mustard derivative can be activated *in vivo* by enzymic splitting to liberate the cytotoxic moiety (Hebborn & Danielli, 1958). One possible interpretation of the present results is that adaptive enzyme formation is involved here. Quantitative measurements have been made colorimetrically to determine the amount of aniline liberated from the analogue by tumour tissue homogenate. A water-soluble dye was produced after coupling with a sulphonated naphthol. Enzymic activity has been demonstrated in potentiated material.

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Presence and Significance of α-Glycerophosphate in Insect Tissue. By F. P. W. WINTERINGHAM. (*Department of Scientific and Industrial Research, Pest Infestation Laboratory, Slough, Buckinghamshire*)

The labelled pool technique has been used to determine the relative concentrations of the principal soluble phosphorus compounds in the adult housefly. One fraction, which was resistant to acid hydrolysis and which represented some 20% of the total acid-soluble phosphorus of the thoracic tissues, was tentatively identified as phosphoglyceric acid by paper cochromatography with the 'authentic' compound but there was an element of doubt because the latter was not chromatographically pure (Winteringham, Bridges & Hellyer, 1955). A mixture of the same insect compound and a sample of glycerophosphate (B.P.), believed to be a mixture of the α- and β-isomers, was almost completely resolved in some instances by paper chromatography and this led to the suggestion that neither isomer was present in detectable quantities (Winteringham, 1958).

The possible importance of α-glycerophosphate in insect metabolism (Sacktor, 1958) prompted a re-examination of the compound recovered from the housefly and the acquisition of chromatographically pure samples of α-glycerophosphate (through the kindness of Dr R. W. Estabrook), β-glycerophosphate, D-2- and D-3-phosphoglycerate (Light and Co.). By a combination of the acid and ammoniacal solvents of Burrows, Grylls & Harrison (1952), and the system of Cowgill (1955) involving the use of molybdate-treated paper, separation of the insect compound (labelled with ^{32}P *in vivo*) from all the known compounds except α-glycerophosphate was achieved on paper chromatograms. Added α-glycerophosphate cochromatographed exactly with the insect compound in all the systems tried. The insect compound was slowly hydrolysed at pH 4 and at 115° (cf. Bailly, 1942). These observations strongly suggest that the insect compound earlier described as phosphoglycerate is in fact α-glycerophosphate. The first sample of glycerophosphate used as a reference apparently contained only the β-isomer.

It follows that experiments on the effects of flight on the distribution of soluble phosphorus in the adult housefly (Winteringham, 1958) suggest a fall in α-glycerophosphate and this effect is indeed consistent with the hypothesis advanced by Sacktor (1958) to explain respiratory metabolism during flight. It also follows that iodoacetic acid and excessive doses of methyl bromide (Winteringham, Hellyer & McKay, 1958) cause a fall in α-glycerophosphate in the adult housefly; any effects on possible undetected traces of phosphoglycerate being unknown.

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DEMONSTRATIONS

Micro-Determination of Zinc in Blood and Other Tissues by Neutron Activation Analysis. By T. E. BANKS, R. TUPPER, E. M. A. WHITE and A. WORMALL. (*Department of Biochemistry and Chemistry, and Department of Physics, Medical College of St Bartholomew's Hospital, London, E.C.* 1)

Neutron bombardment of zinc leads to the production of the radioactive zinc isotope ^{69m}Zn by the reaction ^{68}Zn (n, γ) ^{69m}Zn. This reaction has been made the basis of a method for the estimation of zinc in small amounts of blood (0·1 g.) or other tissues.

The weighed blood or other tissue samples are inserted in polythene pill packs and these packs, together with others containing known amounts of zinc of the same order as that present in the biological samples, are exposed to a high neutron flux in an atomic pile. The irradiated pill packs are dissolved in a mixture of H_2SO_4 and HNO_3 and 'carrier' zinc (about 50 μg.) is added to the solution which is then buffered at pH 5·5. The zinc, including the ^{69m}Zn produced by the irradiation, is then quantitatively extracted by diphenylthiocarbazone solution and freed as far as possible from radioactive contaminants. The amount of ^{69m}Zn is then determined by a scintillation counter in conjunction with a pulse height analyser.

Oxalosis and Primary Hyperoxaluria. By J. C. CRAWHALL, E. F. SCOWEN, A. G. STANSFELD and R. W. E. WATTS. (*The Medical Unit and Department of Pathology, St Bartholomew's Hospital, London, E.C.* 1)

In primary hyperoxaluria, a continuous and probably life-long high urinary oxalate excretion results in progressive renal stone formation with nephrocalcinosis; calcium oxalate deposits occur in some extra renal tissues (oxalosis). We believe that this disease results from an inborn metabolic error which is due genetically to the operation of a rare recessive character.

The present demonstration has been designed: (1) to indicate the clinical features of primary hyperoxaluria, (2) to illustrate the pathological findings in oxalosis, (3) to present the pedigrees of the families upon which our conclusions concerning the genetic features of the disease are based, and (4) to summarize the results of some clinical isotopic tracer studies which indicate that glycine is a major precursor of the urinary oxalate in primary hyperoxaluria. The latter is compatible with the view that the metabolic lesion in primary hyperoxaluria involves an oxidative pathway of glycine metabolism similar to the one described for rat liver by Weinhouse (1955) and by Nakada & Sund (1958).

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **379th Meeting** *of the Society was held at the Department of Chemistry and Biochemistry, St Thomas's Hospital Medical School, Lambeth Palace Road, London, S.E. 1 on Friday, 23 January* 1959. *The following papers were read:*

COMMUNICATIONS

Fibrinolysis in Plasma Fractionation by Heparin. By J. GREEN. (*Paddington Hospital Laboratory Harrow Road, London, W.* 9)

The separation of serum β-lipoproteins as heparin complexes has been reported by Burstein & Samaille (1958) and Scanu, Lewis & Page (1958). During the preparation of fractions from human heparinized plasma, it has been found that the fraction obtained between pH 7·2 and 6·8 at 0·05 ionic strength is spontaneously fibrinogenolytic. This is due to a labile constituent of normal plasma, possibly an active form of the known fibrinolytic enzymes, or an associated kinase.

Spontaneous lysis of the clot formed in the above fraction on addition of commercial thrombin has been found in over 100 random samples. The rate of lysis shows wide individual variation, and is also influenced by the amount of commercial thrombin added. Under standard conditions, lysis times have ranged from a few seconds to about an hour and a half at 37°.

Preparations from fresh plasma show some heparin co-factor activity. The correlation between lytic activity and co-factor is close enough to suggest that in this system the same factor may be concerned.

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The Effect of *o*-Iodosobenzoate on Heart-muscle Succinic Oxidase. By M. B. THORN. (*Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E.* 1)

Slater (1949*a*) used *o*-iodosobenzoate as an oxidizing agent for sulphydryl groups (Hellerman, Chinard & Ramsdell, 1941) when studying the effect of sulphydryl-combining agents on the succinic oxidase system. He concluded that *o*-iodosobenzoate might inhibit the system, not only at the dehydrogenase, but also at a second point, possibly the factor (Slater, 1949*b*) which is destroyed by 2:3-dimercaptopropanol (BAL) in air. This conclusion was based on the observation of differential changes in oxidase and methylene-blue reductase activities after inhibition with *o*-iodosobenzoate, and after reactivation with thiols under anaerobic conditions. Using similar methods, it appeared that *p*-aminophenylarsenoxide affected only the dehydrogenase.

Methylene blue, however, is inefficient, compared with phenazine methosulphate, as a hydrogen acceptor for succinic dehydrogenase, both in soluble and in particulate preparations (see, for example, Singer, Kearney & Massey, 1957). It is therefore likely that changes in succinic dehydrogenase activity will be more correctly demonstrated using phenazine methosulphate rather than methylene blue.

The following experiments have provided no evidence that *o*-iodosobenzoate inhibits the succinic oxidase system anywhere but at the dehydrogenase.

(1) *p*-Aminophenylarsenoxide and *o*-iodosobenzoate inhibited succinate-phenazine reductase and succinic oxidase activities almost equally.

(2) After inhibition by either compound, K_m of succinic oxidase increased slightly. This contrasts with the decrease of K_m which was observed after inhibition by either antimycin or BAL in air (Thorn, 1956), and indicates that the dehydrogenase rather than an intermediate factor was the rate-determining stage.

(3) It is known that succinate, but not malonate, can protect succinic oxidase from inhibition by BAL in air, and that antimycin prevents the protective action of succinate (Thorn & Jackson, 1959; cf. Slater, 1958). Succinate and malonate, however, were equally effective in protecting the system from inhibition by *o*-iodosobenzoate anaerobically, and antimycin had no effect on the protective action of succinate aerobically.

(4) A preparation which had been inhibited by *o*-iodosobenzoate, and reactivated anaerobically

with BAL, regained succinate-phenazine reductase and succinic oxidase activities almost equally, and the oxidase activity was restored to a much greater extent than was demonstrated by Slater (1949*a*).

The inhibitory processes brought about by *o*-iodosobenzoate, or other oxidizing agents, in this preparation are more complex, however, than appears at first sight (cf. Slater, 1949*a*), and secondary effects on another part of the system are not completely ruled out.

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Determination of the Neuraminic Acid Content of Crude Brain-lipid Extracts. By C. Long and D. A. Staples. (*Biochemistry Division, Physiology Department, Institute of Basic Medical Sciences, Royal College of Surgeons of England, London, W.C.* 2)

Böhm, Dauber & Baumeister (1954) determined spectrophotometrically the neuraminic acid content of serum protein after heating with Bial's orcinol reagent and extracting the purple pigment with amyl alcohol. With synthetic *N*-acetylneuraminic acid (Cornforth, Firth & Gottschalk, 1958), we found the absorption spectrum of the reaction product to have a maximum at 570 mμ (ϵ, $6{\cdot}0 \times 10^3$) and a flat portion between 430 and 490 mμ (ϵ, $3{\cdot}8 \times 10^3$). Ox-brain 'strandin' (ganglioside) gave a curve of similar shape, but a solvent-free extract of total ox-brain lipids, under similar conditions, gave a prominent peak at 420 mμ and a shoulder at 650 mμ, in addition to the 570 mμ peak. These anomalies were traced to the galactose residue of the cerebroside present, and made it impossible to determine directly the neuraminic acid content of crude brain lipids.

When the ox-brain lipid extract was partitioned with 0·1M-KCl according to Folch, Lees & Sloane-Stanley (1957), the upper aqueous methanolic phase, after treatment with the orcinol reagent, showed an absorption spectrum rather similar to that of *N*-acetylneuraminic acid or strandin, but with an extra small peak between 430 and 460 mμ, and a shoulder at 640–670 mμ, indicating the presence of some orcinol-reactive substance in addition to strandin.

Folch, Arsove & Meath (1951) have demonstrated the acid-thermolability of the neuraminic acid component of strandin. When *N*-acetylneuraminic acid or strandin was heated at 100° for 30 min. in 4N-HCl, and the orcinol reaction then applied, the absorption maximum at 570 mμ disappeared and was replaced by a new peak at 420 mμ. The 'difference' spectrum (untreated minus acid-treated) showed a single maximum at 570 mμ. A 'difference' spectrum of exactly similar shape was obtained for the upper phase resulting from the Folch *et al.* (1957) partition procedure. Thus is became possible to determine accurately the neuraminic acid content of the upper-phase material by measuring the optical density at 570 mμ of the 'difference' curve. This upper phase contained all the bound neuraminic acid of the original brain-lipid extract, since the lower $CHCl_3$-rich phase gave a difference spectrum showing it to be free from neuraminic acid.

Our thanks are due to Miss Patricia Carroll and Dr G. H. Sloane-Stanley for gifts of synthetic *N*-acetylneuraminic acid and ox-brain strandin, respectively.

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Electron-spin Resonance of Xanthine Oxidase Solutions. By R. C. Bray,* B. G. Malmström and T. Vänngård. (*Institutes of Biochemistry and Physics, University of Uppsala, Sweden*)

Solutions of purified xanthine oxidase have been studied by means of electron-spin resonance (cf. Malmström, Mosbach & Vänngård, 1958). 'Active' and 'inactive' xanthine oxidase samples (Bergel & Bray, 1958) were used, and measurements were made both on the resting enzyme and after treatment either with xanthine or with sodium dithio-

* Permanent address: Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital), London, S.W. 3.

nite. The results obtained are interpreted as indicating that iron is always in the ferrous state, and further that in the presence of the substrate, molybdenum is reduced and FAD free-radicals are formed. While the changes which take place in the presence of the substrate have not at present been proved to be essential for the occurrence of the enzymic reaction, they do nevertheless seem to provide the strongest presumptive evidence which is yet available that molybdenum and also free radicals participate in the oxidation-reduction sequences of xanthine oxidase action.

We thank Dr A. Ehrenberg for carrying out certain confirmatory magnetic susceptibility measurements.

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The Isolation of *n*-Propylmercapturic Acid from the Urine of Animals Dosed with 1-Bromopropane. By T. H. Grenby and L. Young. (*Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E.* 1)

It has been observed that certain alkylating agents can give rise in the animal body to *N*-acetyl-*S*-alkylcysteines, i.e. alkylmercapturic acids. Compounds which have been reported to undergo this reaction *in vivo* are ethyl methane-sulphonate (Roberts & Warwick, 1957, 1958), bromoethane (Thomson, Maw & Young, 1958), 1-bromobutane and certain 1-bromoalkanes higher in the series (Bray & James, 1958). Hitherto evidence for the occurrence of this reaction has been obtained by examination of the urine of the dosed animals by chromatographic and radiochromatographic procedures and by certain quantitative methods, whereas in the present work it has been possible to isolate *n*-propylmercapturic acid from the urine of rats dosed with 1-bromopropane.

$$CH_3 \cdot CH_2 \cdot CH_2 \cdot Br \rightarrow CH_3 \cdot CH_2 \cdot CH_2 \cdot S \cdot CH_2 \cdot CH(NH \cdot CO \cdot CH_3) \cdot CO_2H$$

S-(*n*-Propyl)-L-cysteine and *n*-propylmercapturic acid were synthesized as reference compounds. The former was obtained by allowing L-cysteine to react with 1-iodopropane in anhydrous ammonia, and was converted to *n*-propylmercapturic acid by acetylation with keten or with acetic anhydride. The mercapturic acid was also prepared by the interaction of *N*-acetyl-L-cysteine with 1-iodopropane in anhydrous ammonia.

By means of paper chromatography in two different solvent systems evidence was obtained for the excretion of *n*-propylmercapturic acid by rats, rabbits, guinea-pigs and mice dosed with 1-bromopropane and also by rats dosed with 1-iodopropane. The mercapturic acid was detected by the use of the $K_2Cr_2O_7$-$AgNO_3$ reagent described by Knight & Young (1958), or by the yellow spot produced after spraying the paper with 0·1N-$KMnO_4$.

n-Propylmercapturic acid was isolated from the urine excreted by rats in the first 24 hr. after they had been dosed with 1-bromopropane as a 40% (w/v) solution in liquid paraffin (1·25 g. 1-bromopropane/kg. body wt.). The urine was acidified to pH 2 with HCl and extracted with chloroform. Fractionation of the chloroform extract yielded a crystalline product, m.p. 96–97°, alone and when mixed with authentic *n*-propylmercapturic acid; $[\alpha]_D^{20} - 18°$ in water (*c*, 2%). Found: C, 47·3; H, 7·1; N, 7·4; S, 15·2%. Calc. for $C_8H_{15}O_3NS$: C, 46·8; H, 7·4; N, 6·8; S, 15·6%.

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A Diauxic Effect with *Pseudomonas aeruginosa*. By W. A. Hamilton and E. A. Dawes. (*Department of Biochemistry, University of Glasgow*)

The growth of *Pseudomonas aeruginosa* is supported by a mineral salts medium containing either glucose, citrate, succinate, malate, fumarate or acetate as the sole carbon source. The oxidative pattern of glucose-grown cells towards organic acids has been investigated in connexion with the role of the tricarboxylic acid cycle in the organism (Campbell & Stokes, 1951). However, the behaviour towards glucose of cells grown on organic acids has not been reported.

We have found that such cells (and also cells grown on peptone) display a variable lag period

before glucose is oxidized. Furthermore, when glucose and an organic acid are simultaneously present in the medium, diauxic growth occurs but, in contrast to the previously reported cases with *Aerobacter aerogenes* (Ravin, 1952; Dagley & Dawes, 1953; Davis, 1956), the organic acid is utilized in preference to glucose. This behaviour also extends to some non-proliferating cell suspensions: citrate-grown cells which have been exposed to glucose and are therefore capable of oxidizing both citrate and glucose when these substrates are presented singly, do not attack glucose in the presence of an equimolar concentration of citrate until the citrate concentration has fallen to a low level. This inhibition by citrate is manifest only with cells which are able to metabolize exogenous citrate, i.e. to inhibit, the citrate must have access to the cell. Kleinzeller, Málek, Praus & Škoda (1952) reported a similar effect with glucose and xylose for resting suspensions of a yeast, *Rhodotorula gracilis*, although the present example and also one noted with *Sarcina lutea* (E. A. Dawes & W. H. Holms, unpublished) seem to be the first recorded with bacteria.

An explanation of these findings is being sought in terms of the nature of the enzymes for glucose metabolism, i.e. whether they are constitutive or inducible, and in the possible existence of a permease system (Cohen & Monod, 1957) for glucose.

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Interference by Polyunsaturated Fatty Acids in the Determination of Cholesterol. By D. N. Rhodes. (*Low Temperature Station for Research in Biochemistry and Biophysics, Downing Street, Cambridge*)

Polyunsaturated fatty acids have been found to give spurious colours in the reaction with ferric chloride in concentrated sulphuric and acetic acids, described by Zlatkis, Zak & Boyle (1953) for the determination of cholesterol. The following molar colour yields, measured at the wavelength recommended for cholesterol, were obtained using highly purified materials in the method as described by Henly (1957); the colour yields are expressed as percentages of the cholesterol value: ethyl palmitate 0·047, methyl oleate 0·46, methyl linoleate 2·6, methyl linolenate 6·5, triolein 0·45, trilinolein 2·5 (the last two per fatty acid residue). Significant error will, therefore, be introduced into the estimation of cholesterol by the direct application of the method to lipid mixtures from natural sources where the molar ratio of the polyene acids to cholesterol is high.

The absorption spectra of the linoleate and linolenate complexes differ from each other (maxima at 446 and 478 mμ respectively) and from that derived from cholesterol (maximum at 560 mμ); corrections for the presence of these acids may, therefore, be calculated from extinctions measured at the three wavelengths.

By a suitable choice of conditions a method has been devised for the estimation of diene and triene acids in natural fats free from the higher polyene acids.

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Metabolism of 16β-hydroxyoestrone *in vitro*. By H. Breuer, R. Knuppen and Lieselotte Nocke. (*Chemisches Laboratorium, Chirurgische Universitätsklinik, Bonn-Venusberg, Germany*)

In 1957, Marrian, Watson & Panattoni advanced the hypothesis that 16β-hydroxyoestrone might be the immediate metabolic precursor of 16-*epi*oestriol. Since 16β-hydroxyoestrone has now been isolated from pregnancy urine by Layne & Marrian (1958) it seemed of interest to find whether 16-*epi*oestriol can in fact arise by the metabolic reduction of 16β-hydroxyoestrone.

When 100 μg. of 16β-hydroxyoestrone were incubated with 200 mg. of human liver slices or rat liver slices in Krebs's phosphate saline at 37°, three phenolic metabolites were formed. After sublima-

tion (I) melted at 273° and was indistinguishable from 16-*epi*oestriol by paper chromatography in the systems formamide/chloroform (R_F 0·18) and benzene/methanol/water (100:55:45) (R_F 0·48). After coupling with Fast Black Salt K and paper chromatography of the azo derivative in the system toluene/light petroleum/ethanol/water (Heftmann, 1950), (I) was identical with the spot produced by 16-*epi*oestriol (R_F 0·47). In the sulphuric acid/water reaction (Dirscherl & Breuer, 1954) and in the Kober reaction (Brown, 1952; Bauld, 1954) (I) showed the same absorption curves as pure 16-*epi*oestriol. (II) was formed in smaller amounts (m.p. 237°) and showed the same R_F values as 16-oxo-oestradiol-17β in the system formamide/chloroform (0·32) and benzene/methanol/water (0·70). On reduction with $NaBH_4$, (II) yielded two products which were indistinguishable from oestriol and 16-*epi*oestriol. The Fast Black Salt K derivative of (II) was identical with that produced with 16-oxo-oestradiol-17β (R_F 0·76). Finally, (II) showed the same absorption curves in the sulphuric acid/water reaction and in the Kober reaction as 16-oxo-oestradiol-17β. (III) has not yet been fully characterized.

The quantitative examination revealed that after 30 min. four times as much 16-*epi*oestriol was formed with human liver (24 μg.) as with rat liver (5·5 μg.), whereas the amount of 16β-hydroxyoestrone metabolized was about the same (85 μg.) in both species.

The present findings strongly support the view of Marrian *et al.* (1957) that 16β-hydroxyoestrone can give rise to 16-*epi*oestriol by metabolic reduction. As far as could be determined, the 16-oxo-oestradiol-17β found after incubation could not have been formed artifactually from 16β-hydroxyoestrone. This points to the possibility that 16-*epi*oestriol may have undergone partial oxidation with the formation of 16-oxo-oestradiol-17β.

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Separation of Deoxyribonucleic Acid from Ribonucleic Acid Without the Use of Enzymes.

By K. S. Kirby. (*Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, S.W. 3*)

The separation of deoxyribonucleic acid (DNA) from rat liver by homogenizing in *p*-aminosalicylate solution and extracting with phenol depended upon the use of ribonuclease to remove residual ribonucleic acids (RNA) (Kirby, 1957). Phenyl phosphate and *p*-nitrophenyl phosphate at 0·15M concentration, together with the usual extraction with phenol, were also able to bring about the release of DNA from rat liver. When 2-methyl-1:4-dihydroxynaphthalene diphosphate ('synkavit') or phenolphthalein diphosphate (0·15M) was used to homogenize the tissue a very viscous homogenate was produced, but on shaking with phenol and then centrifuging no DNA was found in the aqueous layer. An insoluble layer which extended into the aqueous layer appeared to contain the DNA when 'synkavit' was used, but with phenolphthalein diphosphate a good separation between the aqueous and phenol layers was achieved and a small amount of insoluble material appeared at the bottom of the centrifuge tube. This insoluble portion contained all the DNA which was brought into solution by several extractions with 20% sodium acetate. After separation from glycogen by extraction with 2-methoxyethanol and phosphate solution the DNA was isolated in about 80–90% yield of that obtained by the *p*-aminosalicylate-phenol method.

The DNA prepared in this manner contained only traces of lysine, arginine, aspartic and glutamic acid and was free from ribonucleic acid, which was present entirely in the aqueous layer. Attempts to separate the RNA have so far resulted in a low yield, but the use of phenolphthalein diphosphate does produce a quantitative separation of DNA from RNA in the case of rat liver without separation of cell nuclei and without the use of enzymes.

Application of this method to rat spleen has shown that a similar separation was achieved, but that it was more difficult to obtain the DNA from the insoluble material, only about 50% being solubilized.

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The Metabolic Conversion of Acenaphthylene into *cis*- and *trans*-Acenaphthene-1:2-diol.

By C. J. W. Brooks, R. P. Hopkins and L. Young. (*Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E.* 1)

When naphthalene (Young, 1947; Booth & Boyland, 1949), anthracene (Boyland & Levi, 1935), and phenanthrene (Young, 1947; Boyland & Wolf, 1950) are administered to animals they are converted in part to dihydrodiols. It has also been established (Brooks & Young, 1956) that indene gives rise *in vivo* to both the *cis*- and *trans*-forms of indane-1:2-diol. In the present work it has been shown that in the rabbit acenaphthylene is converted into *cis*- and *trans*-acenaphthene-1:2-diol (Ring Index numbering). The latter compound can exist in optically active forms.

Benzoates of the two geometrical forms of acenaphthene-1:2-diol were prepared by the action of silver benzoate and iodine on acenaphthylene. With these reactants at room temperature the monobenzoate of the *cis*-diol was formed, whereas at about 80° the product was the dibenzoate of the *trans*-diol. The free diols were obtained by hydrolysis of the esters with KOH in methanol. It was found possible to separate the diols by paper chromatography and to detect them by the fluorescent spots they give in ultraviolet light after the papers have been sprayed with H_2SO_4 in aqueous ethanol and then heated.

Acenaphthylene (0·5 g./kg. body wt.) was administered to rabbits by stomach tube as a 10% (w/v) suspension in 1% (w/v) starch solution. Examination of the urine of the dosed animals by paper chromatography showed the presence of *cis*- and *trans*-acenaphthene-1:2-diol. From the ether extract of the urine (pH 7–8) it was possible to separate an optically inactive crystalline product, m.p. 207–208°, which was identified as *cis*-acenaphthene-1:2-diol by its m.p. and mixed m.p. with the synthetic diol, and also the m.p. and mixed m.p. of its diacetate. A dextrorotatory crystalline product was also separated which consisted of needles with elementary analysis corresponding to acenaphthene-1:2-diol. Its m.p., 158–159°, and its optical rotation in different solvents corresponded to those reported by Jack & Rule (1938) for (+)-*trans*-acenaphthene-1:2-diol. A further dextrorotatory crystalline fraction was obtained which consisted of needles and plates and showed properties to be expected from a mixture of (+)- and (±)-*trans*-acenaphthene-1:2-diol.

From these observations it is concluded that in the rabbit acenaphthylene gives rise to *cis*-acenaphthene-1:2-diol and to a mixture of (+)- and (−)-*trans*-acenaphthene-1:2-diol with the (+)-form preponderating.

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The Effect of 4 MeV Electrons and Heat on the Succinoxidase Activity of Rat Liver.

By Audrey Marples. (*U.K.A.E.A., Isotope Division, Wantage Radiation Laboratory, Grove, Wantage, Berks*)

These experiments were designed to determine the dose required for complete inactivation of enzymes in cells and homogenates and also to investigate the effect of heat on the radiosensitivity of enzymes. The enzyme studied was succinoxidase of rat liver.

Homogenates were prepared by homogenizing the liver with five volumes of ice-cold isotonic sucrose in a stainless-steel Potter–Elvehjem homogenizer. Succinoxidase was determined by the conventional Warburg manometric method (Potter, 1957). Samples were irradiated with 4 Mev electrons from a linear accelerator. In the first set of experiments intact liver slices were irradiated in polystyrene containers placed 1 m. from the accelerator window (dose rate 1·25 Mrads/min.). Containers were kept in ice before and after irradiation and were kept cool during irradiation by blowing cold air over them. In the second set of experiments homogenates were irradiated in slowly rotating test tubes. The temperature was kept relatively constant by playing jets of water of the required temperature on to the tubes. Temperatures used were 15° and 40°. The dose rate for these latter experiments varied from 1·5 to 1·9 Mrads/min. in separate experiments.

Intact liver slices were subjected to a range of doses between 1 and 20 Mrads. In all cases irradiated samples had a strong odour and above 8 Mrads the surface of the liver became pitted. 4 Mrads resulted in 60% inactivation of the enzyme but 20 Mrads were required for complete inactivation.

Homogenates were exposed to a range of doses between 1 and 16 Mrads at 15° and 40°. Irradiation

again resulted in an unpleasant odour and in the evolution of gas at doses of 4 Mrads and greater. 2 and 4 Mrads resulted in 57 and 71 % inactivation respectively, but 16 Mrads were required for complete inactivation of the enzyme. Increasing the temperature during irradiation from 15° to 40° had no significant effect on the sensitivity of the enzyme to irradiation.

These results are in agreement with those of Rajewsky, Gerber & Pauly (1956) using mouse liver, and Green (1955) using ox liver.

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The Effect of Combined Heat and Ionizing Radiation Treatment on the Alkaline Phosphatase Activity of Cow's Milk. By G. Glew. (*U.K.A.E.A., Isotope Division, Wantage Radiation Laboratory, Grove, Wantage, Berks*)

Enzymes in foodstuffs are radiation-resistant and a search has been made for methods of sensitizing enzymes in food to radiation treatment. In a review on the effects of ionizing radiation on enzymes, Marples & Glew (1958) concluded that low doses of radiation (2–5 Mrads) combined with mild heat treatment might be effective in inactivating enzymes in foodstuffs.

Fricke (1952) has shown that egg albumin treated with X-rays has decreased thermal stability, and Clarke (1938) postulated that radiation sensitizes protein to thermal denaturation at lower temperatures than usual. Morgan & Reed (1954), Kempe (1955) and Kan, Goldblith & Proctor (1957) found that radiation can increase the sensitivity of some micro-organisms to heat treatment. Kan *et al.* (1957) suggested that this may be due to the ability of radiation to facilitate protein denaturation.

Fresh, whole Jersey milk was irradiated with γ-rays in a ^{60}Co source or with electrons in a 4 Mev linear accelerator. The alkaline phosphatase activity was estimated using a modification of the method described by King & Wootton (1956) using Folin and Ciocalteu's reagent. The phosphatase inactivation curve for electron-irradiated milk is exponential and the enzyme is completely inactivated at approximately 25 Mrads. Because of the radio-resistance of the enzyme the possibility of lowering the inactivation dose by combination of heat and radiation treatment was investigated.

Milk was irradiated with ^{60}Co γ-rays at doses of 0·25–2·0 Mrads. Samples were heated at 50° for 15 min. either before or after irradiation. The degree of inactivation of phosphatase in those samples heated before irradiation was not significantly different from that in unheated, irradiated samples. The percentage of the enzyme inactivated was significantly increased ($P = 0{\cdot}005$) by heating after irradiation. The effect of heating at 20° and 40° during irradiation with doses of 0·25–4·6 Mrads was also investigated. Irradiation at 40° significantly increased ($P = 0{\cdot}025$) inactivation of the enzyme compared with 20°.

Compared with heat or irradiation treatments alone, mild heat treatment applied during or after irradiation effectively increases the amount of enzyme inactivated. There is no indication that a combination of the heat and radiation treatments described will completely inactivate the enzyme at sterilization doses of radiation (2–5 Mrads).

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Crystals of Riboflavin making up the Tapetum Lucidum in the Eye of the Lemur, *Galago crassicaudatus*. By A. Pirie. (*Nuffield Laboratory of Ophthalmology, Oxford*)

The tapetum lies behind but adjacent to the retina. It forms a reflector, so that any light that is not absorbed during the first passage through the light-sensitive cells is reflected back again and hence has a second opportunity of acting as a visual stimulus (Walls, 1942). The tapetum of the lemurs is bright yellow and occupies the whole central area of the fundus (Johnson, 1901). The yellow material which makes up the tapetum of the nocturnal lemur *Galago crassicaudatus* appears to consist of crystals

of riboflavin. These crystals lie immediately behind the pigment epithelial cells of the retina (which are non-pigmented in the tapetal area) so that when the retina is removed from an open unfixed eye the tapetum appears like a sheet of beaten gold. The material can be extracted in water or 1% formic acid giving a yellow solution with green fluorescence. The absorption spectrum shows maxima at 266, 370 and 445 mμ, corresponding with those of riboflavin. Mr Rosen, St Mary's Hospital, kindly measured the fluorescence and found it corresponded exactly with that of riboflavin. The tapetal material can be distinguished from riboflavin phosphate both by chromatography and by its solubility in benzyl alcohol. All other properties examined agree with those of riboflavin except the crystal form of the substance in the eye.

Typical yellow needle crystals arranged in wheat-sheaves are deposited from formic acid solutions on standing but material examined in smears from the eye shows only plate-like crystals. Plate forms of riboflavin have been noted by Sakate (1956). To resolve the question whether the plate crystals in the eye contain a compound or complex of riboflavin with some other substance Dr D. M. Hodgkin has kindly examined them by X-ray crystallography and finds them to be identical with one of the plate forms of riboflavin obtained by recrystallization of a commercial preparation. Crystals of riboflavin occur in the cells of a fungus *Eremothecium ashbyii* (Guilliermond, 1941) but have not previously been described in tissues of higher animals.

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An Effect of Oral Hydrogen Peroxide on the Blood of Tumour-Bearing Rats. By GRACE M. JEFFREE. (*University of Bristol Pathology Research Laboratory, 18 Guinea Street, Bristol, 1*)

Blood catalase and haemoglobin were estimated weekly on tail blood of forty rats bearing the Walker carcinoma, twenty of which were treated by replacing their drinking water with 0·45% (w/v) hydrogen peroxide from the sixth day after introduction of the tumour. The tumour regressed in fourteen of the treated animals and seventeen controls (Jeffree, 1958). In all rats, both catalase and haemoglobin levels declined as the tumour grew in size and rose again as it regressed, showing a roughly inverse relationship between tumour weight and log catalase or log haemoglobin. Blood catalase was approximately proportional to haemoglobin, as was found by Miller (1958) in human anaemias; but an increased catalase/haemoglobin ratio, of borderline significance, was found in treated animals after the first fortnight of peroxide treatment, together with a wider scatter of values. On analysis, this increased catalase/haemoglobin ratio was found to be due to the values in five rats only, the other nine surviving treated rats showing ratios similar to those of controls. Absolute values could not be compared during the period of tumour regression, when both catalase and haemoglobin were regenerating from low levels; but, when values obtained after apparently complete regression of the tumour were compared, it was found that blood catalase values of these five animals were similar to those of controls, but haemoglobin levels were considerably lower. It is thought therefore that administration of oral hydrogen peroxide at this concentration has no effect on the blood catalase of tumour-bearing rats, but may diminish the production of haemoglobin in susceptible animals. This effect appears to have no bearing upon tumour regression, which had generally already begun before it was manifest. Catalase and haemoglobin estimations during the period of growth of the tumour were of no value in predicting the likelihood of regression.

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The Incorporation of Labelled Amino Acids into Protein by the Isolated Cytoplasmic Membrane of *Bacillus megaterium*. Intermediate Reactions. By P. Brookes, A. R. Crathorn and G. D. Hunter. (*Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London, S.W.* 3)

Butler, Crathorn & Hunter (1958) showed that the site of protein synthesis in *B. megaterium* was probably located on or closely associated with the cytoplasmic membrane. Membranes isolated *in vitro* were capable of incorporating a variety of labelled amino acids into the TCA-precipitable fraction at rates comparable with those observed in membranes in the intact protoplast, and this incorporation process has now been studied in greater detail. Partial and total acid hydrolysates of the labelled TCA-precipitable material from which lipids and nucleic acids had been removed were subjected to two-dimensional paper chromatography followed by radioautography. It was found that all the radioactivity could be accounted for by the original amino acid added, and that partial hydrolysates contained several labelled peptides. A further indication that the process studied involved true protein synthesis was afforded by inhibition studies using chloramphenicol, which gave results similar to those observed when using the intact cells. Finally, it was found that the membrane fraction could catalyse the activation of a variety of amino acids, as measured by ^{32}P-pyrophosphate-ATP exchange.

Ultrasonic radiation disintegrated the membranes into two fractions, one sedimentable and one 'soluble', roughly equal in amount. It was found that the specific radioactivity of the protein derived from the 'soluble' fraction was about six times as great as that found in the sedimentable fraction in most experiments. The activation enzymes were present in the 'soluble' fraction. Extraction of the 'soluble' fraction with phenol also gave, after fractionation (Kirby, 1956), RNA carrying labelled amino acid. The amino acids could be removed by treatment with 0·02 N-alkali (30° for 30 min.) or by ribonuclease, and the role of this labelled RNA in the incorporation process is at present being investigated. However, in short-term labelling experiments, the radioactivity in the sedimentable fraction was about equal to that in the 'soluble' fraction. When the fractions are labelled separately only the sedimentable fraction has been found to be capable, although very inefficiently, of amino acid incorporation into protein. Part of this TCA-precipitable radioactivity is removable by lipase or boiling methanol, and lipids may play an important part in the incorporation process.

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DEMONSTRATION

An Automatic Recording Scanner for Radiochromatograms. By A. R. Morrison and L. Young. (*Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E.* 1)

This apparatus has been constructed in order to make possible the automatic recording of the amount and distribution of radioactivity on strip chromatograms. It consists of a mechanism for passing the paper strip under an end-window Geiger–Müller tube connected to a ratemeter, the meter of which has been replaced by a recording milliammeter.

The radiochromatogram is drawn at a fixed rate under the Geiger–Müller tube by being wound on a spool while it is held taut by a 50 g. weight attached to its free end. The spool is rotated by a motor (Type RQR, Drayton Regulator and Instrument Co. Ltd., West Drayton, Middlesex) running at 30 rev./hr. and coupled through two gear boxes which allow selection of a speed of 3, 12, or 30 in./hr. for the rate of movement of the radiochromatogram. The radiochromatogram passes close to the end-window of the Geiger–Müller tube and is separated from it by a slotted metal plate which is removable. Three such plates are available with slot widths of 0·2, 0·5 and 1·0 cm. thereby permitting variation in the resolution obtained in the scanning process. As the apparatus is normally used for scanning radiochromatograms carrying ^{35}S, the Geiger–Müller tube employed (EHM 2 S, General Electric Co. Ltd., London) has a thin mica window (1·7 mg./cm.^{2}). The output from this tube is fed into a ratemeter (Type 5054, Panax Equipment Ltd., Redhill, Surrey). This instrument is connected to a d.c. pen-recording milliammeter (Evershed and Vignoles Ltd., London), the record chart of which can be moved at 3, 12 or 30 in./hr. As the radiochromatogram and the chart can travel at the same speed it is possible to compare the two side by side after scanning has been completed. This comparison is facilitated by marking the beginning and end of the radiochromatogram, prior to scanning, with spots of ink to which some radioactive material has been added.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **380th Meeting** *of the Biochemical Society was held in the Beveridge Hall of the Senate House, University of London, on Saturday,* 14 *February* 1959, *when the following papers were read:*

SYMPOSIUM ON THE BIOSYNTHESIS AND SECRETION OF ADRENOCORTICAL STEROIDS

Adrenocortical Steroids: their Separation, Identification and Estimation. By I. E. Bush. (*Radcliffe Infirmary, Oxford*)

Steroid biochemistry owes most of its efflorescence of the last ten years to the discovery of the medical uses of cortisone and related steroids. These advances have almost all depended upon new techniques for separating, identifying and estimating steroids. The most important of these are partition chromatography, infrared spectroscopy, and the use of radioactive labelling.

Partition chromatography was at first difficult to apply to steroids because of their low solubility in water and strong adsorption to paper. After relatively abortive attempts to use water-soluble derivatives or special methods such as rubber as supporting material for organic stationary phases, it was found possible to use Celite, paper and silica gel with systems based on aqueous alcohols, formamide or glycols. Adsorption chromatography was the basis of the isolation of adrenal steroids (Reichstein) and of much work on urinary steroids (Callow, Dobriner, Dingemanse). Partition methods have been the basis of most recent work on adrenal steroids, microbiological metabolism of steroids, oestrogens, androgens, cardiac glycosides and aglycones, bile salts and bile acids, and the urinary steroid metabolites. It is now possible to effect all separations achieved by absorption methods by using suitable partition systems.

An important feature of steroid chromatography is due to the enormous number of possible compounds: this is the danger of mis-identification if chromatographic properties are relied on to the exclusion of others. This is dealt with by using simple microchemical reactions such as acetylation or oxidation with chromic acid or bismuthate, a method introduced by Zaffaroni's group.

Infrared spectra have been extensively studied and a wide range of correlations between structure and absorption bands has been established (Jones). Ultraviolet absorption spectra in sulphuric acid (Zaffaroni, Bernstein) and alcoholic alkali (Meyer) are very useful techniques: in conjunction with chromatography and comparison with reference substances, they provide identifications almost as reliable as by infrared spectra. Oxidations with bismuthate (Norymberski), the reaction with phenylhydrazine-sulphuric acid (Porter & Silber), the standardization of the Köber method (Bauld & Brown), and the fluorescence reaction with sodium hydroxide (Bush) have been valuable additions to the range of chemically specific methods of identifying and estimating steroids.

Unpublished work has shown that useful generalizations about the behaviour of steroids in partition systems can be made using Martin's theory and the R_M function of Bate-Smith and Westall. These have led to the development of several new families of solvent systems which will be discussed. The rationale of identification of steroids by chromatography and of the development of quantitative chromatographic methods will be discussed in the light of this theoretical approach.

The Biosynthesis of Adrenocortical Steroids. By J. K. Grant. (*Department of Biochemistry, University of Edinburgh*)

The application of a variety of techniques has clearly established that adrenocortical steroids may be formed in mammalian organisms from acetate or cholesterol and certain pathways of biosynthesis leading to the C_{21} steroid hormones are well defined. Outstanding questions of major interest include the possibility of alternative pathways excluding cholesterol and the role of ascorbic acid. Differences may exist between biosynthetic pathways in the adrenals of species with lipid stores in fasciculata cells and those without such stores, and little attention appears to have been paid to possible functions of cholesterol esters in the biosynthetic process.

Hydroxylations of cholesterol and progesterone play a prominent part in the biosynthesis of adrenocortical steroids. These reactions occur at different sites in the cell and require the direct intervention of molecular oxygen. In this respect the enzymes involved resemble certain other oxygen-requiring enzymes such as non-specific liver hydroxylase, phenolase, kynurenine hydroxylase and squalene oxycyclase, grouped together by Mason. These also require an electron donor, frequently reduced triphosphopyridine nucleotide. The nature of the steroid hydroxylating enzymes and their possible modes of action will be discussed.

The C-20, 21 and C-17, 20 desmolases, yielding pregnenolone and 17-oxo steroids respectively, have been less fully investigated, and the biosynthetic pathways leading to the formation of oestrogens in the adrenal are not yet clear. The present state of knowledge of these topics will be reviewed.

Recent work on the inhibition of adrenocortical steroid biogenesis will be discussed.

The Morphology of the Adrenal Gland. By T. SYMINGTON. (*Department of Pathology, Royal Infirmary, Glasgow, C* 4)

Adrenal glands can be divided roughly into two main groups, those containing large amounts of lipid and those containing very little lipid visible to the naked eye. The former group is characteristic of diverse groups of animals, ranging from man and monkey on the one hand to rats and guinea-pigs on the other. Glands showing very little lipid invariably occur in ruminants. The two groups of glands differ in their reaction to stress. When the gland contains large amounts of lipid, a feature of the reaction to stress is the depletion of the lipid. This occurs with certain variations in the adult and in the child human gland and in the rat and monkey. In addition to the depletion of lipid, morphological and enzymic changes occur particularly in the human gland. In the ruminant adrenal no obvious change is seen in the morphology and histochemistry of the gland in the reaction to stress. Similar changes occur in the human gland following ACTH administration and these will be described, as well as the theories on the site of formation of steroid hormones.

In investigations of the steroid content of the adrenal glands, so-called ketosteroid stains have been developed which have been said to demonstrate adrenal steroids. These methods will be discussed in the light of results from the extraction and isolation of the steroid hormones from the adrenal. Observations have been made which indicate that the one type of adrenal cell is capable of forming C_{21}, C_{19} and possibly C_{18} adrenal hormones and the importance of the so-called compact cell in this connexion will be discussed. The observations made on the pathological physiology of the gland may be applied to the problems of adrenal pathology.

The Relation of Steroid Secretion to the Histological Zones of the Adrenal Cortex. By P. J. AYRES.* (*Courtauld Institute of Biochemistry and Department of Physics Applied to Medicine, Middlesex Hospital Medical School, London, W.* 1)

Histological and physiological studies on the adrenal gland led to theories on the correlation of structure and function of the cortex. It was observed that the cortex consisted mainly of three distinctive concentric zones, the zona glomerulosa, fasciculata, and reticularis. When the adrenal gland is removed there occurs imbalance of carbohydrate, water, and electrolyte metabolism, and the steroids secreted by the cortex capable of maintaining life after adrenalectomy have overlapping effects as glucocorticoids and mineralocorticoids. Therefore, the problem was whether the zones once formed are independent and have different secretory functions, or whether the zones are related to one another in varying degrees as may be their secretory capacities.

It was suggested by Swann (1940) that there may be a division of secretory function between the zones of the adrenal cortex, and that these functions were not equally dependent on the hypophysis, since although adrenalectomy was followed by electrolyte imbalance, the imbalance did not occur after hypophysectomy, where the zona fasciculata atrophied but the zona glomerulosa remained unchanged for some time. The results obtained in rats by Deane & Greep (1946) supported the concept that an electrolyte active steroid was secreted by the zona glomerulosa, independent of the pituitary but subject to change according to

* External Staff Medical Research Council.

electrolyte intake. Further indirect evidence was to come after the isolation of the potent mineralocorticoid aldosterone by Simpson & Tait (1952) from whole adrenal extract. Singer & Stack-Dunne (1954) showed by the analysis of blood in rats that on a low-potassium diet the aldosterone secretion was reduced, but that of corticosterone remained unchanged; also hypophysectomy was found to have a much greater effect on corticosterone than on aldosterone. More recently it was shown that in dogs (Farrell, Banks & Koletsky, 1956) after hypophysectomy the zona fasciculata atrophied and the secretion of cortisol and corticosterone fell, whereas the zona glomerulosa remained unaffected and the aldosterone secretion was maintained. All this indirect evidence indicated that a salt-retaining hormone, more specifically aldosterone, is preferentially produced by the zona glomerulosa and the glucocorticoid, specifically cortisol in the dog and corticosterone in the rat, by the zona fasciculata.

The first direct evidence was obtained by incubation of tissue from the ox adrenal cortex (Ayres, Gould, Simpson & Tait, 1956). The results showed that aldosterone was preferentially produced by the zona glomerulosa and cortisol by the zona fasciculata. The values obtained for corticosterone on an equal tissue weight basis were similar for both zones. These results indicate that in the ox adrenal cortex the 11-hydroxylase system is equally divided per unit weight between the two zones and the cortisol and aldosterone results indicate the 17-hydroxylase to be in the zona fasciculata only and the 18-oxidase system confined to the zona glomerulosa. These results were independently confirmed by others using rat adrenals (Giroud, Stachenko & Venning, 1956).

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The Secretion of Sex Hormones by the Adrenal Gland. By R. V. Short. (*A.R.C. Unit of Reproductive Physiology and Biochemistry, University of Cambridge*)

It has been recognized for many years that some tumours of the adrenal gland may give rise to symptoms of excessive masculinization or feminization. Subsequent work has shown that androgens, oestrogens and progestogens are all present in adrenal gland extracts, and as evidence for the secretion of these compounds, 17-oxo steroids, oestrogens and pregnanediol have been isolated from the urine of gonadectomized patients. With the advent of chromatographic techniques it became possible to demonstrate the synthesis of androgenic, oestrogenic and progestational steroids *in vitro*, using homogenates of adrenal tissue, or perfusates of excised glands. For obvious technical reasons, comparatively few studies have been made of the steroids present in adrenal venous blood *in vivo*, and the present communication will be concerned mainly with evidence of this nature, derived from experiments on man and animals.

Androgens. Four androgens have been reported in human adrenal vein blood, namely androstenedione, 11β-hydroxyandrostenedione, androsterone and dehydro*epi*androsterone. Of these four, androstenedione is undoubtedly the most active biologically, and it may be present in relatively high concentrations. In cattle, androstenedione, androsterone and dehydro*epi*androsterone cannot be detected even in large samples of adrenal venous blood. 11β-Hydroxyandrostenedione is probably present in the adrenal venous blood of the cow, sheep, rat and cat, although it seems to possess relatively little biological activity.

Oestrogens. Oestrogens have only been looked for in human adrenal vein blood, and the amounts present are too small to permit detection by existing methods.

Progestogens. There is evidence for the presence of at least three progestogens in adrenal venous blood, namely progesterone, 17α-hydroxyprogesterone and 20α-hydroxypregn-4-en-3-one. Progesterone has been detected in the adrenal venous blood of the human, cow, sheep and pig. 17α-Hydroxyprogesterone is probably present in the adrenal venous blood of the human and the cow. Whilst this steroid shows little biological activity in rabbit bio-assays, it is known to be 60 times as active as progesterone in the Hooker Forbes assay. 20α-Hydroxypregn-4-en-3-one has recently been detected in the adrenal venous blood of the young calf, although it has never been found in the adult animal.

At the present time the physiological significance of the sex hormones secreted by the adrenal gland is not understood. However, the secretion of these steroids may be of considerable importance in certain pathological conditions.

The Control of the Secretion of Adrenocorticosteroids. By M. Vogt. (*Department of Pharmacology, University of Edinburgh*)

The dependence of the size of the adrenal cortex on pituitary activity was discovered early in the 1920's; when, a few years later, Smith described a good method of extirpating the pituitary in the rat, it became also possible to study the dependence of adrenocortical secretion on pituitary hormones. We now know that the amount of corticosterone and cortisol secreted by the mammalian adrenal is controlled by corticotrophin (ACTH) elaborated by the anterior lobe. The question of adaptation of the secretion of these two corticoids to the needs of the body has therefore become a question of how the pituitary obtains the information necessary to adjust the activity of the cells of the adrenal cortex to changes of environmental or endogenous demands. It is known that neural mechanisms are involved, and that there may be more than one way of increasing secretion of ACTH. The present views on the subject will be presented.

The fact that salt metabolism is less disturbed in the hypophysectomized mammal than carbohydrate and protein metabolism led to the suggestion many years ago that secretion of the adrenal 'mineralocorticoid' is less dependent on the pituitary than the secretion of glucocorticoids. With the identification of the mineralocorticoid as aldosterone, it has became possible to attack the question of the control of mineralocorticoid secretion directly. It has been confirmed that dependence on the pituitary is either absent or much less pronounced for aldosterone than it is for corticosterone and cortisol. Several factors calling for increased or decreased production of aldosterone have been discovered, and much research is being carried out on the afferent and efferent pathways involved in changes of aldosterone secretion. The present position of our knowledge will be reviewed.

The Mode of Action of Corticotrophin from the Biochemical Point of View. By N. Saba. (*Ministry of Agriculture, Veterinary Laboratory, New Haw, Weybridge, Surrey*)

It has been shown that the major steroid end products of the adrenal are either corticosterone or cortisol or a mixture of both (Bush, 1953). The conversion of both cholesterol and acetate to corticosterone and cortisol has been demonstrated in isolated, perfused, bovine adrenals (Zaffaroni, Hechter & Pincus, 1951).

From the results of the Worcester Foundation group, where various substrates labelled with ^{14}C were perfused through isolated bovine adrenals, Hechter (1951) proposed a sequence of reactions for the conversion of cholesterol to corticosterone and cortisol. The evidence at present indicates that acetate may be converted to corticosteroids by a route which does not involve cholesterol as an obligatory intermediate.

ACTH stimulates the conversion of cholesterol to corticosterone and cortisol but has little, if any, effect on acetate conversion (Stone & Hechter, 1954). It was also demonstrated that ACTH acts upon a specific site of the cholesterol pathway—namely, the conversion of cholesterol to progesterone (Stone & Hechter, 1954).

Haynes & Berthet (1957) observed that ACTH-stimulated adrenals showed increased phosphorylase activity. From this and subsequent studies they have postulated that ACTH causes increased production of reduced triphosphopyridine nucleotide, which is then used in the hydroxylation reactions of the steroid biosynthetic sequence.

Saba & Hechter (1955) found that adrenal mitochondria could convert cholesterol to progesterone. They also found that mitochondria contain cholesterol which is easily available for conversion to progesterone. Further investigations on adrenal mitochondria have led them to suggest that ascorbic acid may inhibit cholesterol metabolism not only by acting directly on the appropriate enzymes but also by making some of the cholesterol biochemically 'inert', i.e. the mitochondrial cholesterol may exist in more than one pool (Hayano, Saba, Dorfman & Hechter, 1956). It was also suggested that the spatial relationships of the enzymes of the biosynthetic sequence may play some part in the mechanism of ACTH action.

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